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Bioanalysis of picomole amounts of acetylcholine by ion-pair partition chromatography applied to rat sciatic nerve

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A method for determination of acetylcholine in small, discrete biological objects by use of ion-pair technique has been developed. Acetylcholine is extracted as an ion pair with 3,5-di-t-butyl-2-hydroxybenzenesulphonate and separated from co-extracted components by ion-pair partition chromatography with picrate as the counter ion and porous cellulose as support. The quantitative evaluation is made from the acetylcholine peak in the chromatogram obtained by ultraviolet detection. Acetylcholine has been analysed in 1 cm large pieces of rat sciatic nerve containing about 60 pmol (10 ng). The overall recovery of the method is 100 ± 10% at the 120 pmol level of acetylcholine in a sample.

The determination of acetylcholine and choline in biological material is attracting continuous interest and recently a handbook of chemical assay methods was introduced (Hanin, 1974). Lately, sensitive analytical methods have been presented based on gas-chromatography with mass spectrometric detection (Stavinoha & Weintraub, 1974; Hanin & Skinner 1975).

Ion-pair extraction and liquid-liquid chromatography were combined in an analytical procedure for acetylcholine in rat brain enabling the determination of 500 ng in a sample (Eksborg & Persson, 1971). The present paper reports an analytical method based on the same principles where the sensitivity is increased by about 50 times. This was achieved by selecting another extraction agent and adapting modern liquid chromatography with high-sensitive on-line ultraviolet detection with a response linear to concentration (cf. Eksborg & Schill, 1973).

MATERIALS AND METHODS

Apparatus. The liquid chromatograph consisted of a Chromatronix 'Cheminert' Metering Pump CMP-1L and a Chromatronix Model 200L photometer with a 8 μl flow cell and operating at 253·7 nm. The separation columns were of borosilicate glass (length 30 cm; i.d. 2·7 mm) and silanized before use and they were equipped with Chromatronix injection tees and connectors. The precolumn was 5/8″ o.d. and 10 mm i.d. with a length of 25 cm. The whole chromatograph was placed under thermostated conditions with a temperature of 22·0 ± 0·1°.

Chemicals and reagents. Methylene chloride, chloroform and l-pentanol were of Fisher certified ACS quality. Diethyl ether was obtained from Mallinckrodt and perchloric acid and phosphate buffer substances from E. Merck. Methylene chloride was freshly distilled and saturated with water before use.

Acetylcholine and choline were used as the iodides from Sigma Chem. 3,5-di-t-butyl-2-hydroxybenzenesulphonate (BPS) as the sodium salt was kindly synthesized by Brändström (1974).

Tetrabutylammonium, tetraethylammonium and acetylcholine picrates in crystalline form were obtained from our chemical department. An aqueous solution of neutral pH containing picrate and the quaternary ammonium compound was extracted with methylene chloride which was evaporated to give a crystalline salt.

Cellulose, ethanolysed, (Munktell 410) was extracted with ethanol to remove traces of pyridine.

Column preparation. The porous cellulose powder used as support was mixed with the stationary phase, 0·06 M picrate in phosphate buffer solution pH 6·5, 1·0 ml per 4 g of support. The mixture was suspended in the mobile phase, 5% of 1-pentanol in chloroform, which had been equilibrated with the stationary phase. After homogenization for 1 min the slurry was transferred to the column in small portions and packed by a rod. The filling was covered with the mobile phase during the packing procedure. The precolumn was filled by the same technique. The chromatographic system was equilibrated by passing the mobile phase through the column for more than 1 h at a flow rate of 0·2 ml min^{-1}.

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Extraction constant. An aqueous solution of BPS, 0·01 M in 0·1 M NaH_2PO_4 containing acetylcholine (ACh) in the range 10^{-4}–10^{-3} M was equilibrated with methylene chloride in centrifuge tubes for 30 min. BPS was measured in the organic phase by spectrophotometry and the extraction constant of the ion pair was computed according to equation 1.

$$E^{x}_{AChBPS} = C_{AChBPS_{org}} \times (C_{ACh_{aq}} \times C_{BPS_{aq}})^{-1} \quad .. \quad .. \quad .. \quad (1)$$

No variation in the magnitude of the extraction constant was observed, which means that side reactions have no significant influence in the concentration range examined. The value of log E^{x}_{AChBPS} = 2·42 was obtained.

Determination of acetylcholine in rat sciatic nerve

(1) The sciatic nerve tissue (1–1·5 cm) is rapidly removed from the rat, and homogenized as soon as possible in 1·0 ml of ice-cold 0·1 M perchloric acid in a small glass homogenizer.

(2) The homogenate is centrifuged in a refrigerated centrifuge at 15 000 rev min^{-1} for 20 min and the supernatant is decanted. The precipitate is rinsed once with 0·5 ml 0·1 M perchloric acid which is combined with the first supernatant in a centrifuge tube.

(3) The perchloric acid solution is mixed with 0·3 ml of 0·5 M Na_2HPO_4 and extracted for 1–2 min with 4·0 ml of diethylether by shaking.

(4) After centrifugation at 2000 rev min^{-1} for 5 min the aqueous phase is transferred to a new centrifuge tube, 2·0 ml of 0·01 M BPS in 0·1 M NaH_2PO_4 is added and the mixture is extracted with 4·0 ml CH_2Cl_2 by shaking for 2 min.

(5) After centrifugation at 2000 rev min^{-1} for 5 min the methylene chloride phase is transferred to a tapered centrifuge tube containing 200 μl of 0·01 M tetrabutylammonium picrate in CH_2Cl_2. The extraction is repeated once and the two methylene chloride phases are combined.

(6) After evaporation of the methylene chloride under nitrogen the residue is dissolved in 100 μl of the mobile phase.

(7) 80 μl is injected with a syringe into the separation column containing 0·06 M picrate (pH 6·5) as the stationary phase and 5% of 1-pentanol in $CHCl_3$ as the mobile phase. The acetylcholine picrate peak is eluted at 0·4 ml min^{-1} after about 15 min.

(8) Quantitation is achieved by peak area measurement and comparison with the acetylcholine picrate peak of an injected standard in the mobile phase.

The analytical procedure enables the determination of 60 pmol of acetylcholine in a sample with a standard deviation of 10–15 %.

RESULTS AND DISCUSSION

Extraction. The supernatant perchloric acid solution from the homogenization of the nerve tissue was buffered to a pH of about 6 to improve the stability of acetylcholine. The purification by extraction with diethyl ether decreased the front peak in the chromatogram significantly.

The concentration of BPS used in the ion-pair extraction of acetylcholine was determined by its limited solubility. The counter ion concentration, the magnitude of the extraction constant and approximately equal phase volumes would give a theoretical recovery of about 50% for one extraction and about 75% for a repeated extraction (Schill, 1974). The experimental studies showed, however, that acetylcholine was extracted quantitatively in the analytical procedure (> 99%). The improved extraction ability is probably due to dissociation of the ion pair in the organic phase, which is of particular importance at low concentrations (Modin, Persson & Schill, 1971; Lagerström, Westerlund & Borg, 1972).

No dissociation of the acetylcholine–BPS ion pair was observed in the partition studies for determination of the extraction constant, since the concentrations were too high. A dissociation constant of $10^{-5.20}$ was determined for the ion pair between tetrabutylammonium and anthracene-2-sulphonate and methylene chloride as the organic phase (Westerlund & Borg, 1970). A constant of the same magnitude can be expected for acetylcholine—BPS, since the binding forces between the ion-pair components are similar, which would give theoretical support for the increased extraction recovery in the analytical method.

Liquid-liquid chromatography

The chromatographic system for isolation of acetylcholine from other components in the biological extract was identical with that used in the previous acetylcholine method (Eksborg & Persson, 1971). Picrate is a suitable counter ion for acetylcholine both as regards the magnitude of the extraction constant and the high response and sensitivity for a modern on-line chromatographic ultraviolet detector (Eksborg & Persson, 1971; Eksborg & Schill, 1973). 2 ng of acetylcholine will be detectable with a retention time of about 15 min.

Ethanolysed cellulose as support for the stationary phase was found to be superior to other medium-

sized particles for ion-pair partition chromatography of organic ammonium compounds concerning both column efficiency and peak symmetry (Eksborg & Schill, 1973). Recent studies in ion-pair partition chromatography have shown that micro silica particles as support improve the column efficiency considerably (Kraak & Huber, 1974; Persson & Karger, 1974; Knox & Jurand, 1975; Wahlund, 1975; Lagerström, 1976). So far, however, no reports have been presented with this packing material in the separation of quaternary ammonium ions as ion pairs with hydrophobic anions and preliminary studies indicate that thorough investigations are necessary before such systems can be introduced.

The high selectivity of ion-pair extraction has been demonstrated in several studies, as recently discussed in a review (Schill, 1974). This is also valid for the ion-pair system used in the chromatographic isolation of acetylcholine and is illustrated in Fig. 1 by the separation of acetylcholine from propionylcholine (added) in a rat brain extract. These two compounds

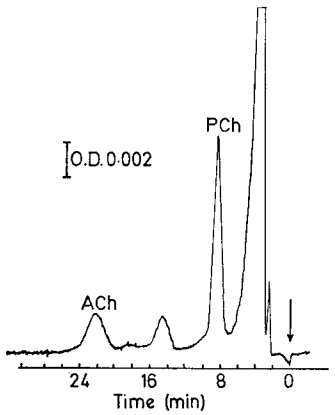

FIG. 1. Separation of acetylcholine (endogenous) and propionylcholine (added) in extract from 1/40 of a rat brain. Support: Cellulose Munktell 410. Stationary phase: Na-picrate 0·06 M in phosphate buffer pH 6·5. Mobile phase: chloroform–pentanol (19 + 1) 0·4 ml min.$^{-1}$ Sample: acetylcholine (ACh) 180 pmol, propionylcholine (PCh) 400 pmol.

are homologues, differing by one methylene group, and a separation factor of about 3 is obtained between the two peaks, which is in accordance with earlier observations (Gustavii, 1967). Propionylcholine should be a potential internal standard in the determination of acetylcholine in biological material provided that it is not interfered with too much by the front peak in the chromatogram.

In the extract from the biological object acetylcholine is present as an ion pair with BPS, which is a more hydrophobic anion than picrate. If no precautions are taken on injection of the sample, acetylcholine will migrate partly as the ion pair with BPS, the concentration of which will decrease along the column, and partly as the picrate, which has a constant concentration. A discontinuous partitioning process is obtained and will result in double peak formation or a distorted acetylcholine peak (Eksborg & Schill, 1975). Tetrabutylammonium picrate was added in high concentration as a displacer to the biological extract to prevent acetylcholine migrating in a form other than as the picrate ion pair. Tetrabutylammonium is assumed to reduce the available concentration of BPS, by elution as ion pair in the front peak, to a level where disturbances are absent.

A change in the properties of the chromatographic column was observed on injection of the first biological sample. The retention time of acetylcholine-picrate was decreased by 5–10% compared with a pure reference sample on the newly packed column. After that there was no further change in the retention time and the column could be used for more than 50 biological samples. It can be assumed that a small portion of BPS remains in the column after the injection of a biological extract, since there is a large excess of BPS compared with acetylcholine due to co-extraction of other sample components. BPS moves down the column slowly and will finally influence the retention time and detector response. In many instances, however, it was possible to regenerate the column by injection of 50 μl of the mobile phase containing tetraethylammonium picrate (0·03 M), which is slightly retained in the column and seems to exchange the BPS for picrate.

Rat sciatic nerve does not seem to contain much choline, since no peak for choline picrate was observed in the chromatogram from a nerve tissue extract. However, choline is present in much higher concentration than acetylcholine in most biological materials like rat brain. In such an instance four samples could be injected consecutively before choline picrate from the first injection started to elute from the column. An interval of more than 1 h was then necessary to elute all four choline peaks. Preliminary experiments with extracts from rat brain or muscle tissue containing large amounts of choline showed that injection of 50 μl of the mobile phase containing dipicrylamine (10^{-2} M as the acid) removed choline from the column within 15 min thus reducing the delay time significantly. Choline is extracted from the column as the ion pair with dipicrylamine, the rest of which is eluted as the acid from the column without causing any noticeable change of the column system.

Quantitative determination

Peak area measurement was compared with peak height measurement and electronic integration in the quantitative evaluation of the amount of acetylcholine from the chromatogram. Peak area measurement gave a lower value of the standard deviation on repeated injection of a test solution of pooled extracts and was selected as the evaluation method.

The recovery and reproducibility of the method of determination were checked by analysing spiked and pooled sciatic nerve samples which were divided into several indentical portions each containing 120 pmol of acetylcholine. The standard deviation was 10% and the recovery measured in relation to an injected standard solution was 100%.

A large number of authentic sciatic nerve samples were analysed according to the method of determination and were found to average about 60 pmol of acetylcholine per cm of nerve tissue. Fig. 2 is a chromatogram from an authentic sample.

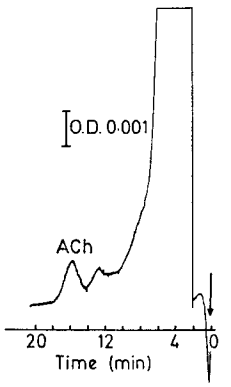

FIG. 2. Determination of acetylcholine (ACh) in a 1 cm piece of rat sciatic nerve. Support: Cellulose Munktell 410. Stationary phase: Na-picrate 0·06 M in phosphate buffer pH 6·5. Mobile phase: chloroform–pentanol (19 + 1) 0·4 ml min^{-1}. Sample: Extract from authentic nerve tissue containing 60 pmol acetylcholine (ACh).

Conclusions

Acetylcholine was analysed down to the 60 pmol concentration in biological material by a method of determination based on ion-pair technique. Rat sciatic nerve was the analytical object and by the use of an inexpensive liquid chromatograph at least 10 samples a day could be handled by one person. The method presented seems to be fully competitive with most other analytical techniques that have been suggested for sensitivity, selectivity and technical ease. Acetylcholine was also determined in pieces of rat brain and the method should be applicable to other biological materials with minor modifications.

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