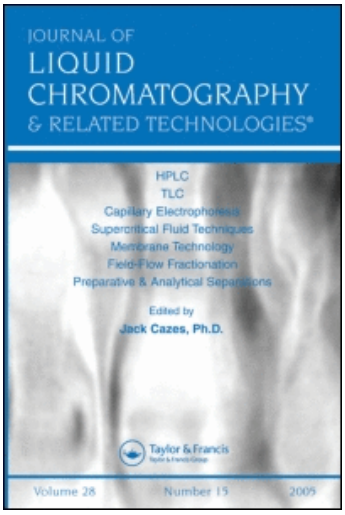

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Improved Method for Determination of Acetylcholine, Choline, and Other Biogenic Amines in a Single Brain Tissue Sample Using High Performance Liquid Chromatography and Electrochemical Detection

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To cite this Article Beley, Alain , Zekhnini, Abderrahman , Lartillot, Serge , Fage, Dominique and Bralet, Jean(1987) 'Improved Method for Determination of Acetylcholine, Choline, and Other Biogenic Amines in a Single Brain Tissue Sample Using High Performance Liquid Chromatography and Electrochemical Detection', Journal of Liquid Chromatography & Related Technologies, 10: 13, 2977 — 2992
To link to this Article: DOI: 10.1080/01483918708066840
URL: http://dx.doi.org/10.1080/01483918708066840

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(13), 2977-2992 (1987)

IMPROVED METHOD FOR DETERMINATION OF ACETYLCHOLINE, CHOLINE, AND OTHER BIOGENIC AMINES IN A SINGLE BRAIN TISSUE SAMPLE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL DETECTION

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ABSTRACT

A simple method for determination of ACh, Ch, NA, DA, 5-HT and their related metabolites on the same brain tissue sample was developed by HPLC-ED. The electrochemical detection system is equipped with a platinum electrode for ACh and Ch detection, or a glassy carbon electrode for CA and 5-HT detection. ACh and Ch can be separated with bonded silica or polystyrene reverse phase columns, using a pH 7 mobile phase. They are converted to H_2O_2 by the passage of the effluent through an in line post column

reactor with covalently bonded ACh esterase and Ch oxidase. This step ensures sensitivity, reliability and enzyme economy. Tissue preparation consists of formic acid/acetone extraction and purification by tetraphenyl boron exchange with high reproductible recoveries. The time necessary for the whole procedure is short, making it well adapted to large series. CA, 5-HT and related metabolites can be simply analysed on an aliquot of the tissue extract.

INTRODUCTION

High performance liquid chromatography coupled to electrochemical detection (HPLC-ED) was first proposed by Potter et al. (1) for analysis of pmole quantities of acetylcholine (ACh) and choline (Ch). The method is based on ACh/Ch reverse phase separation, followed by enzymatic conversion of the eluates by means of continuous ACh esterase and Ch oxidase addition to the effluent. The resulting hydrogen peroxide is detected electrochemically.

However, while usable, the method is not suited to routine analysis, because of the high consumption of enzymes. To overcome this problem different attempts were made in order to immobilize the enzymes on post column reactors by ion exchange (2,3) or by covalent bonding (4-7). Chromatographic conditions differ according to the authors. Ion exchange silica (4) or reverse phase silica (1,5-9) analytical columns are subject to degradation at the alcaline pH necessary for optimal enzyme activity. Mobile phases with pH < 7 need post column buffer addition via a second pump (1,5-9); this can be source of base line instability. Polystyrene reverse phase columns (2,3) are compatible with alcaline mobile phases, but are potentially less resistant to compression than silicas. Another problem concerns ACh/Ch extraction from tissue. Simple techniques, e.g. direct analysis of unpurified perchloric (4) or formic acid/acetone extracts (8), may be complicated (7) by interfering responses due

to catecholamines (CA). ACh/Ch Reineckate salt precipitation (1-3) is more selective, but time-consuming and leads to poor and variable recoveries.

Future analysis of ACh/Ch by HPLC-ED depends on workable methods for routine analysis. Our purpose was to develop a rapid, reliable and low cost assay, compatible with the analysis of CA, 5-hydroxytryptamine (5-HT) and their related metabolites on the same sample of tissue. We investigated different procedures and were able to obtain satisfactory results using different commercial reverse phase columns and a post column reactor with covalently bonded enzymes. Tissue extracts were purified with high reproductible recoveries by means of a rapid organic phase extraction procedure.

METHODS

Reagents

ACh chloride, Ch chloride, ACh esterase type III (electric eel), Ch oxidase (Alcaligenes), noradrenaline (NA) bitartrate, dopamine (DA) hydrochloride, 5-HT oxalate, 5-hydroxyindole acetic acid (5-HIAA) and 3-4 dihydroxybenzylamine hydrochloride (DHBA, internal standard for CA and 5-HT) were purchased from Sigma Chemical (St Louis, MO, U.S.A.). Ethylhomocholine bromide (EHCh, internal standard for ACh and Ch) was synthetized (1) from 3-dimethyl-amino-1-propanol (Aldrich, Strasbourg, France) and bromoethane (Aldrich). All other chemicals were reagent grade. Porous glass beads (200-400 mesh, pore size 350 $\mathring{A}$) were purchased from Sigma Chemical.

Apparatus

A liquid chromatographic system from Waters Associates (Milford, MA, U.S.A.) was used. The electrochemical detector

(Chromatofield, Chateauneuf-les-Martigues, France) was equipped either with a platinum electrode (Bioanalytical Systems, W Lafayette, IN, U.S.A.) for ACh, Ch assay, or with a glassy carbon electrode (Chromatofield) for CA, 5-HT assay. The oxidation potential was + 0.5 V for ACh and Ch, and + 0.8 V for CA and 5-HT versus an Ag/AgCl electrode.

Enzyme Reactors

Two reactors were comparatively tested. In the first case the enzymes were adsorbed (2,3) on a 3 cm 10 µm Aquapore AX 300 anion exchange cartridge (Brownlee Labs, Santa Clara, CA, U.S.A.). Ch oxidase (100 U) was dissolved in 2 ml of mobile phase and injected onto the cartridge via a teflon tube connected to the cartridge holder. After washing the cartridge with the mobile phase, ACh esterase (100 U) was adsorbed according to the same procedure.

In the second case, Ch oxidase and ACh esterase were immobilized covalently with glutaraldehyde onto alkylamino glass beads (10, 11). Porous glass beads were heated in 5% (V/V) nitric acid at 100°C for 1 min, washed with water, dried and heated at 115 °C for 12 h in toluene containing 10% (V/V) of 3-aminopropyltriethoxysilane. They were coupled with glutaraldehyde by resuspending for 2 h at room temperature in a 2% (W/V) glutaraldehyde aqueous solution. ACh esterase and Ch oxidase (100 U) were dissolved in 1 ml of 0.05 M phosphate buffer pH 7 and immobilized covalently by periodical shaking with 120 mg of the activated beads. The glass beads containing the immobilized enzymes were used to pack a 2 mm i.d. x 2 cm reactor.

Chromatographic Conditions

- ACh and Ch

Two bonded silica reverse phase columns (Spherisorb ODS-2 5 µm, 4.6 mm i.d. x 15 cm from Biochrom, Champniers, France, and

Hypersil ODS 5 µm, 4.6 mm i.d. x 25 cm from Gynkotek, München, Germany) and two polymeric reverse phase columns (PLRP-S 5 µm, 4.6 mm i.d. x 15 cm from Polymer Labs, Shropshire, U.K:, and PRP-1 5 µm, 4.1 mm i.d. x 15 cm from Hamilton, Reno, NV, U.S.A.) were tested for ACh and Ch analysis. They were protected either by C 18 (Waters Associates) or PRP-1 (Brownlee) guard cartridges. The different columns required different mobile phases for complete separation of ACh, Ch and EHCh. These phases consisted of 0.1M KH_2PO_4 containing 0 to 50 mg/l of sodium octane sulfate and 150 (polymeric columns) or 500 to 700 mg/l (bonded silicas) of tetramethylammonium chloride (TMA). Final pH was adjusted to 7 and sometimes to 8 (polymeric columns) with 5N KOH.

In order to minimize enzyme losses, the assays with the anion exchange reactor were performed using a lower salt concentration elution system (2,3).It consisted of 20 mM TRIS-HCl buffer at pH 7 containing 10 mg of sodium octane sulfate and 150 mg/l of TMA.

- CA, 5-HT and related metabolites

The separation of CA, 5-HT, and related metabolites was performed using a Radial Nova-pack C 18 cartridge from Waters Associates (4 µm, 8 mm i.d.x 10 cm) equipped with a C 18 guard cartridge. The mobile phase consisted of 0.1M KH_2PO_4 adjusted to pH 4 with 5N H_3PO_4, containing 40 mg/l of EDTA, 1.37 mg/l of sodium heptane sulfate and 12% (V/V) of methanol.

Tissue Preparation

Male Sprague Dawley rats weighing about 300 g were divided into three groups. The rats of the first group were decapitated and the brains quickly removed and dissected on ice cold glass. The cerebral hemispheres and brain stem were immediately frozen in liquid nitrogen. The brains of the second group of rats were frozen with liquid nitrogen using the in situ freezing technique

(12) which allows blood perfusion of the cerebral tissue until freezing time. The rats of the third group were decapitated and the heads immediately frozen in liquid nitrogen. The brains from both in situ frozen and frozen head groups were chiselled out and dissected in the frozen state during liquid nitrogen irrigation. All specimens were stored at - 80 °C until ACh/Ch extraction.

The extraction procedure is summarized in Fig. 1. The cerebral tissue was sonicated with 10V of N formic acid/acetone (15V/85V) containing the internal standards EHCh and DHBA. After centrifugation at 20,000 g at 4°C, 0.5 ml of the supernatant were transferred into a conical centrifugation tube and vortexed with 2 ml of heptane/chloroform (8V/1V). The organic layer was removed and 15 µl of the aqueous fraction were injected into the CA/5-HT chromatographic system. ACh, Ch and EHCh were extracted from the remaining aqueous fraction by tetraphenyl boron exchange (13) into 250 µl of 3-heptanone containing 3 mg/ml of sodium tetraphenyl boron. Two hundred µl of the upper layer were transferred into a second conical tube. ACh, Ch and EHCh were finally extracted with 50 µl of N HCl. The hydrochloric extacts were dried under vacuum after removing the organic layer. Dried samples can be stored at - 80 °C until analysis. They were dissolved in the mobile phase immediately before injection into the ACh/Ch chromatographic system.

RESULTS AND DISCUSSION

Chromatographic Conditions for ACh and Ch Analysis

Satisfactory separation of ACh, Ch and EHCh could be obtained using the four columns tested. However, compression of the stationary phase and rapid loss of efficiency was observed with some PLRP-S columns, even when used below the pressure limit

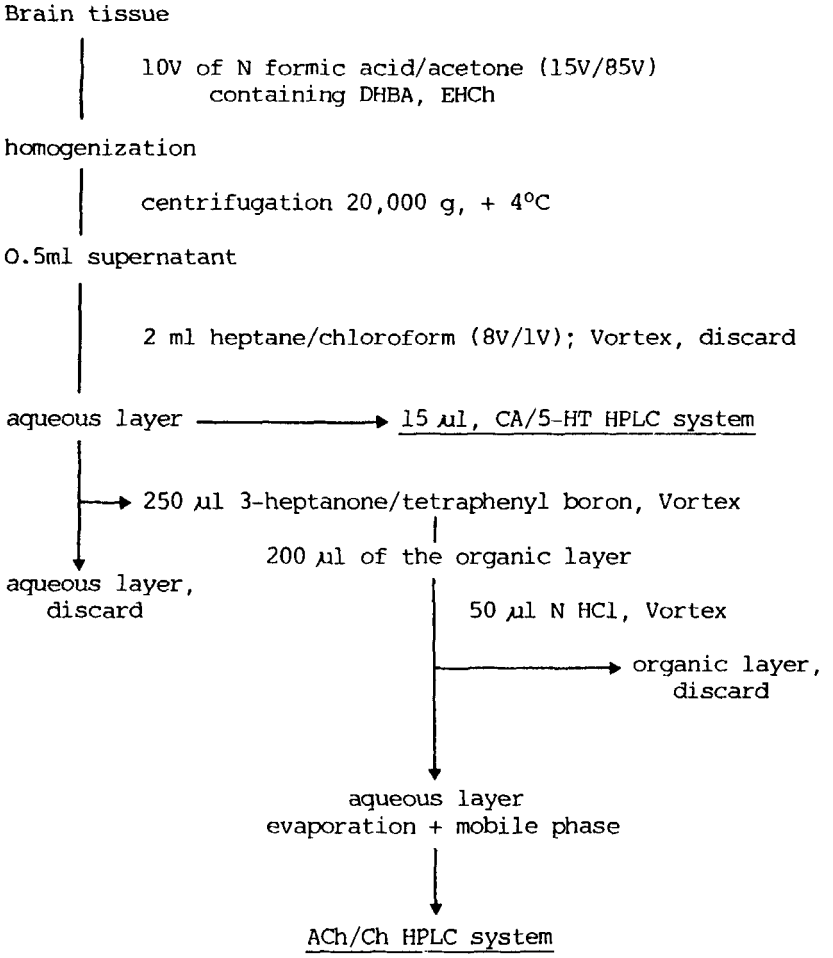

FIGURE 1. Flow chart for the extraction procedure.

indicated by the manufacturer. With bonded silica columns, final pH 7 of the mobile phase is a compromise between optimal enzyme function and stability of the column material, which dissolves at pH > 7. Polymeric supports allow the use of pH 8 mobile phases, corresponding to optimal activity of the enzymes (5). However, increasing the pH to 8 was not found to improve the sensitivity under the conditions tested (glass bead reactor, 200 pmoles injected), probably due to the large quantities of immobilized enzymes.

As it has been often reported previously, ACh was found to adsorb strongly to bonded silica supports, resulting in broad, tailing peaks. TMA, which competes with ACh and Ch for the stationary phase, improves the peak shape. Enzyme inhibition was reported for TMA concentrations 100-200 mg/l, when ACh esterase and Ch oxidase were added to the effluent (1). However, higher concentrations can be used with post column reactors (4). Under our experimental conditions, peak tailing was satisfactorilly reduced by increasing TMA up to 500-700 mg/l, without affecting the sensitivity, since the peak heights were increased.

Enzyme Reactors

Ion exchange and activated glass bead reactors retained enzyme activity to a different extent. After ion exchange immobilization, acceptable retention of Ch oxidase activity was observed for 3 weeks, but ACh esterase activity did not exceed 1 week. Due to continuous ACh esterase elution, daily correction for ACh sensitivity was necessary by insertion of standards between the assays.

Glass bead reactors were recently described (5), using a different chromatographic system with an acid mobile phase which necessitates post column addition of buffer in order to allow enzyme activity. In this type of reactor long spacer arms for enzyme immobilization garantee high activity of the enzymes.

Covalent links between enzymes and the support are extremely stable (5) and virtually free of breakdown. Our experience is that the same reactor, tested daily with 200 pmoles of ACh, provides a response similar to its original response after 3 months of utilization at room temperature with continous circulation of the eluent. Peak heights of Ch are approximatively twice and four times that of the same number of pmoles of ACh and EHCh. The reproducibility of the method tested by repeated injection of the same sample (n=6), indicated intra assay coefficients of variation of 1.1% for ACh, 2.5% for EHCh and 2.7% for Ch. Sandard curves, presented in Fig. 2, show the linearity of the detection. The sensitivity of the method was essentially restricted by the noise level of the baseline and only slightly influenced by the composition of the mobile phase. Routinely, we can quantify 1 pmole of Ch and 2 pmoles of ACh. Higher sensitivity, up to 0.3 and 0.6 pmoles, can be obtained under optimal conditions.

Tissue Analysis

- ACh and Ch

Extraneous endogenous compounds may interfere with ACh and Ch (1,7), necessitating tissue extract purification. Tetraphenyl boron exchange was chosen for removing ACh and Ch for the following reasons: a) the isolation procedure is simple and short (about 20 min), b) it avoids manipulation of insoluble derivates of ACh and Ch, which can be a source of product loss, c) ACh and Ch samples are not diluted. Recoveries were 87.4 $\pm$ 4.0 % for ACh, 76.9 $\pm$ 4.1 % for Ch and 66.4 $\pm$ 2.8 % for EHCh (means $\pm$ SEM, n = 7). Injections of tissue extracts give sharp solvent fronts (Fig. 3 B); no extraneous peaks were found when a reactor filled with uncoupled glass beads was installed (Fig. 3 C).

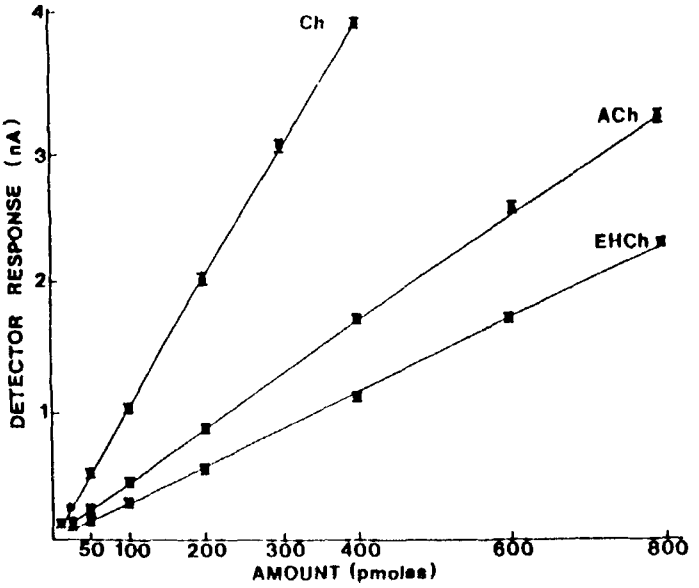

FIGURE 2. Calibration curves for ACh, Ch and EHCh. Standards of ACh, Ch and EHCh were injected into the analytical system equipped with the covalently bonded enzyme reactor (see Methods). Mobile phase, 0.1M KH_2PO_4 pH 7 containing 10 mg/l of sodium octane sulfate and 600 mg/l of TMA; flow rate, 1 ml/min. Analytical column, Gynkotek Hypersil ODS 5 µm. Data are the means ± SEM, n = 3.

ACh and Ch contents in the three groups of rats are presented in Table 1. Freezing of the brain in situ is a suitable technique for measurements of labile cerebral metabolites. Table 2 shows, for example, the values obtained for cerebral phosphocreatine (PCr), ATP and glucose after freezing of the brain in situ, and after freezing of the head immediately after decapitation (14). PCr, ATP and glucose values were much lower in the frozen head group, showing that in situ freezing is the only valuable technique for these labile metabolites. In the present work, ACh and Ch levels did not differ significantly (Table 1)

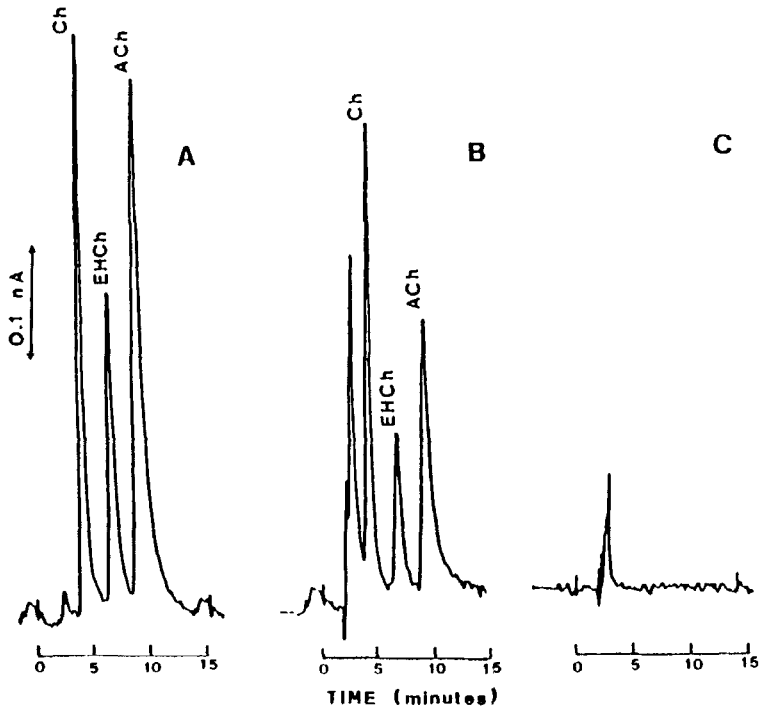

FIGURE 3. HPLC analysis of ACh and Ch.
A, standard sample containing 40 pmoles of Ch, and 80 pmoles of ACh and EHCh, 20 µl injected; B, extract of the cerebral hemisphere from a frozen rat head containing 70 pmoles of EHCh, 30 µl injected; C, injection of 15 µl of the same extract using an inactive post column reactor. Chromatographic conditions as described in Fig. 2.

between frozen head and in situ frozen samples, and were in the same range as those reported after microwave irradiation (5,7,8). Therefore, these two freezing techniques appear suitable for ACh/Ch analysis, whereas decapitation and ice cold dissection showed a tendency to lowered ACh values, reaching - 32% in the brain stem ($p < 0.05$, when compared to the in situ frozen group). However, the most remarkable difference consisted in 2-3 times

TABLE 1

ACh and Ch content (nmole/g) in rat brain

Groups of rats	Cerebral Hemispheres ACh	Cerebral Hemispheres Ch	Brain stem ACh	Brain stem Ch
In situ frozen	36.5 ± 1.8	29.9 ± 2.1	25.8 ± 1.0	23.1 ± 2.7
Frozen head	33.5 ± 3.3	33.1 ± 1.6	24.4 ± 3.2	22.9 ± 1.1
Decapitated	29.0 ± 3.7	71.9 ± 2.8*	17.6 ± 3.4*	76.8 ± 15.2*

Data are the means ± SEM, n = 6. Brains from the decapitated group were frozen in liquid nitrogen after cerebral dissection on ice cold glass. Brains from the in situ frozen group were frozen with intact blood circulation (12). Animals of the frozen head group were decapitated and the heads immediately frozen in liquid nitrogen. Cerebral structures of both in situ and frozen head groups were dissected in the frozen state.
* $p < 0.05$ versus the in situ frozen group.

TABLE 2

Influence of brain freezing conditions on the cerebral labile metabolites PCr, ATP and glucose.

	Metabolite content (μmole/g)	
	In situ frozen group	Frozen head group
PCr	4.75 ± 0.15	1.02 ± 0.14 *
ATP	3.03 ± 0.14	2.30 ± 0.12 *
Glucose	4.70 ± 0.45	0.30 ± 0.05 *

Data are the means ± SEM, n = 8. The freezing procedures are described in Table 1. Quantification of metabolites was performed in the cerebral cortex dissected in the frozen state. * $p < 0.01$.

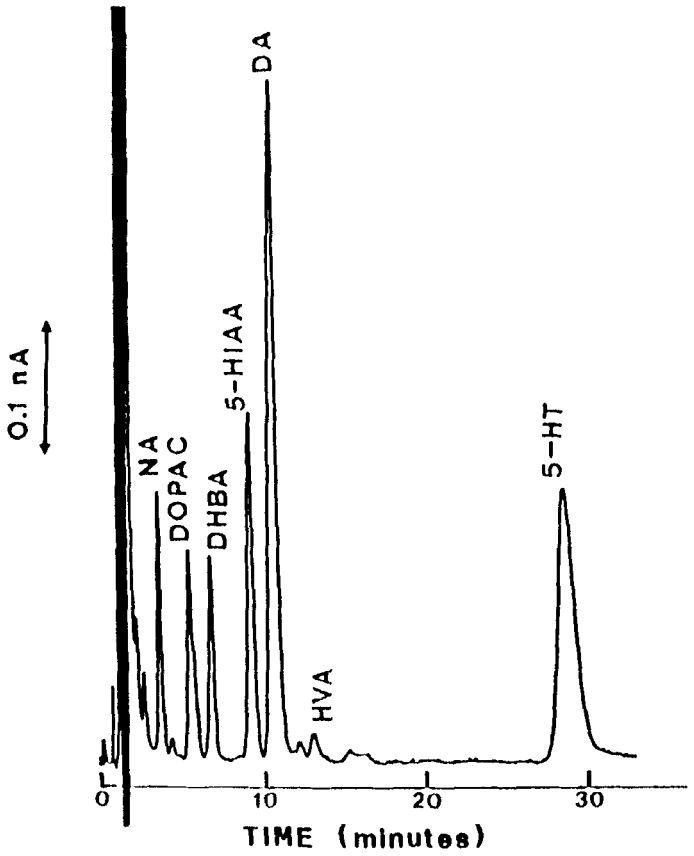

FIGURE 4. HPLC analysis of CA, 5-HT and metabolites. Injection, 15 µl of tissue extract of the cerebral hemisphere from a frozen rat head containing 3.5 pmoles of DHBA. Mobile phase, 0.1M KH_2PO_4 pH 4 containing 40 mg/l of EDTA, 1.4 g/l of sodium heptane sulfate and 12% of methanol; flow rate, 2 ml/min. Analytical column, Radial Nova-pack C18 4 µm.

increase of Ch. Ten fold increase of Ch was reported previously after decapitation (8). Such increases cannot result from ACh post mortem hydrolysis, but may reflect alterations of phosphatidylcholine metabolism.

- CA, 5-HT and related metabolites

Measurements were carried out in the cerebral hemispheres of the frozen head group of rats. Fig. 4 shows a typical chromatogram obtained by injecting an aliquot of the formic acid/acetone extract into the CA/5-HT system as described in Methods. Means in nmole/g ± SEM (n = 6) were as follows: NA, 2.16 ± 0.11; DA, 7.01 ± 0.25; DOPAC, 1.21 ± 0.17; HVA, 0.88 ± 0.15; 5-HT, 2.85 ± 0.14 and 5-HIAA, 2.45 ± 0.25.

In conclusion, the procedure developed in the present work offers several advantages which are useful for ACh/Ch analysis by HPLC-ED. The choice of an in line post column reactor with extremely stable enzyme immobilization ensures reliability and low cost of the assay. High immobilized enzyme activity allows the use of mobile phase pH compatible with many commercial columns without affecting the sensitivity. Therefore, supplementary equipment for post column buffer addition is not necessary. Extraction of ACh and Ch, and further purification by tetraphenyl boron exchange with high reproductible recoveries is rapid , simple and well adapted to large series. Another advantage is that the procedure is compatible with CA and 5-HT measurements on the same extract.

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