ANALYTICAL BIOCHEMISTRY 151, 182–187 (1985)

#625

High-Performance Liquid Chromatography of Water-Soluble Choline Metabolites

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Received June 7, 1985

We have developed a new method for the separation of [^{3}H]choline metabolites by high-performance liquid chromatography. Using this method it is possible to separate, in one step, all of the known major water-soluble choline metabolites present in crude acid extracts of cells that have been incubated with [^{3}H]choline, with baseline or near-baseline resolution. We use a gradient HPLC system with a normal-phase silica column as the stationary phase, and a linear gradient of increasing polarity and ionic strength as the mobile phase. The mobile phase is composed of two buffers: Buffer A, containing acetonitrile/water/ethyl alcohol/acetic acid/0.83 M sodium acetate (800/127/68/2/3), and buffer B (400/400/68/53/79), pH 3.6. A linear gradient from 0 to 100% buffer B, with a slope of 5%/min, is started 15 min after injection. At a flow rate of 2.7 ml/min and column temperature of 45°C, typical retention times for the following compounds are (in min): betaine, 10; acetylcholine, 18; choline, 22; glycerophosphocholine, 26; CDP-choline, 31; and phosphorylcholine, 40. This procedure has been applied in tracer studies of choline metabolism utilizing the neuronal NG108-15 cell line and rat hippocampal slices as model systems. While the compounds labeled in the NG108-15 cells were primarily phosphorylcholine and glycerophosphocholine, reflecting high rates of phospholipid turnover, in the hippocampal slices choline and acetylcholine were the major labeled species. Identification of individual peaks was confirmed by comparing the elution profiles of untreated cell extracts with extracts that had been treated with hydrolyzing enzymes of differing specificities. This HPLC method may be useful in studies of acetylcholine and phosphatidylcholine metabolism, and of the possible interrelationships of these compounds in cholinergic cells.

KEY WORDS: HPLC; metabolites; choline; acetylcholine; phosphatidylcholine.

The metabolic fate of choline in cholinergic neurons and other cells may be investigated by tracing the incorporation of choline into its various cellular metabolites. Indeed, radioisotopically labeled choline has been widely used in studies of acetylcholine turnover and release and of the metabolism of membrane choline-containing phospholipids. This approach requires a method for the separation of intact radiolabeled choline from the compounds to which it is converted. Existing techniques such as paper chromatography (1,2), high-voltage paper electrophoresis (3–7), various modes of ion-exchange chromatography (2,7–10), and thin-layer chromatography (11,12) either are time consuming or require multiple extraction or prepurification steps, and thus are inconvenient. Moreover, none of these methods is capable of separating all of the known cellular metabolites of choline and thus all invariably provide poor resolution of some of these compounds.

HPLC methods for the separating and quantifying of compounds of biological origin have become popular in the last decade because they offer the inherent advantages of liquid chromatography in a highly efficient implementation. Numerous methods, involving ion-paired reverse-phase HPLC, have been described for separating quaternary amines

0003-2697/85 $3.00

(13–16), including one for separating choline from acetylcholine (17). However, these assays provide inadequate retention of phosphorylated choline metabolites and thus are unsuitable for studies requiring a complete separation of choline metabolites.

We have therefore developed a new normal-phase HPLC method for the one-step separation of all of the major water-soluble choline metabolites present in crude cellular extracts.

MATERIALS AND METHODS

HPLC system. Our gradient HPLC system (Rainin, Woburn, Mass.) is equipped with two Rabbit HP pumps, a pressure monitor, and an LC-22 temperature controller (the latter from Bioanalytical Systems, West Lafayette, Ind.). The pumps are controlled by an Apple IIe microcomputer running a gradient control program (Gradient Manager, Model 702 version 1.2, (c) by Gilson International). A normal-phase Rainin Microsorb-silica column (5 μm spherical particle size, 4.6 mm i.d. $\times$ 250 mm length) has been used as the stationary phase. The mobile phase was composed of two buffers: Buffer A, containing acetonitrile/water/ethyl alcohol/acetic acid/0.83 M sodium acetate (800/127/68/2/3), pH 3.6, and buffer B (400/400/68/53/79), pH 3.6. A linear gradient from 0 to 100% buffer B, with a slope of 5%/min, was started 15 min after injection. The gradient profile is shown in Fig. 1. Flow rate was 2.7 ml/min and column temperature was maintained at 45°C. Fractions (2.7 ml) of the eluate were collected in scintillation vials, 10 ml of Hydrofluor (National Diagnostics, Somerville N. J.) was added to each vial, and radioactivity was then determined by liquid scintillation spectrometry in a Beckman LS-7500 spectrometer with an efficiency of 25-35% for ^{3}H and 70% for ^{14}C.

Radioactive standards. [*methyl*-^{3}H]Choline chloride (80 Ci/mmol), [*methyl*-^{14}C]cytidine diphosphocholine (42 Ci/mol), and [*acetyl*-^{14}C]acetylcholine iodide (2.3 Ci/mol) were all purchased from New England Nuclear, Boston Massachusetts. [*methyl*-^{3}H]Glycerophosphocholine was prepared in our laboratory from [*methyl*-^{3}H]phosphatidylcholine (20–40 Ci/mmol, New England Nuclear) by mild alkaline hydrolysis as described (18). [^{3}H]Betaine was prepared from [^{3}H]choline in the reaction catalysed by choline oxidase. Sodium phosphate buffer (0.1 ml, 0.2 M pH 7.8), containing 1 μmol of [^{3}H]choline (10 Ci/mol) and 0.08 units of choline oxidase (Sigma, St. Louis, Mo.) was incubated for 1 h at 30°C. This procedure resulted in the total conversion of choline to betaine, as HPLC analysis of the product revealed only one radioactive peak and no counts at the position of a [^{3}H]choline standard. [*methyl*-^{3}H]Phosphorylcholine was synthesized in the reaction catalyzed by choline kinase in the presence of ATP and magnesium. A solution (0.1 ml) containing 7.5 mM glycylglycine (pH 8.5), 11 mM magnesium chloride, 0.6 mM ATP, 1 nmol of [^{3}H]choline (100

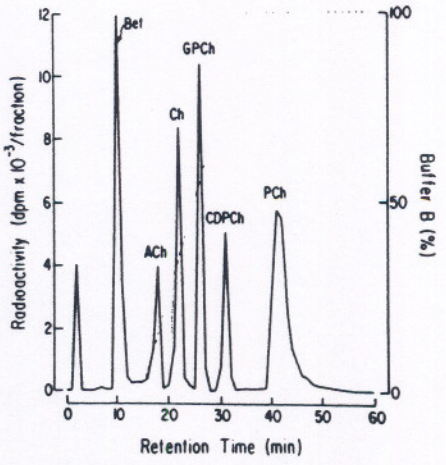

FIG. 1. HPLC separation of radiolabeled standard water-soluble choline metabolites. A mix of six radioisotopically labeled standards (20 μl) was chromatographed as described under Materials and Methods. Briefly, two buffers were used: Buffer A (acetonitrile/water/ethyl alcohol/acetic acid/0.83 M sodium acetate, 800/127/68/2/3) and buffer B (400/400/68/53/79). A linear gradient from 0 to 100% buffer B (slope = 5%/min) was started 15 min after injection. Flow rate was 2.7 ml/min and column temperature was 45°C. The standards utilized in this separation were: Bet, [^{3}H]betaine; ACh, [^{14}C]acetylcholine; Ch, [^{3}H]choline; GPCh, [^{3}H]glycerophosphocholine; CDP-Ch, [^{14}C]cytidinediphosphocholine; PCh, [^{3}H]phosphorylcholine. The dashed line delineates the gradient's profile.

Ci/mol), and 0.01 unit of choline kinase (Sigma) was incubated for 30 min at 30°C. Unreacted choline was extracted to 1.5% tetraphenylboron in 3-heptanone. As shown by HPLC, the aqueous phase was free of choline radioactivity. The radioactivity present in this phase eluted as a single peak which could be completely converted to choline by alkaline phosphatase (data not shown).

Cell culture and labeling. NG108-15, a neuroblastoma × glioma hybrid cell line, was kindly provided by Dr. M. Nirenberg, NIH, Bethesda, Maryland. Cells (passages 17–25) were subcultured in 6 × 35-mm multiwell plates at a concentration of 150,000 cells/well and grown under an atmosphere of 90% air/10% CO_2 in Dulbecco's modified Eagle's medium (GIBCO, Grand Island N. Y.) containing 0.1 mM hypoxanthine, 1 μM aminopterine, 16 μM thymidine (all from Sigma), and 5% fetal calf serum (GIBCO). After 24 h growth medium was replaced with a serum-free N2 medium (19) with 10 μM choline and the cells were further incubated for 48 h. The cells were then labeled by incubation with 60 μM [^{3}H]choline (2 μCi/ml, 33 μCi/μmol) in N2 medium for 48 h. Following incubation the radioactive medium was removed and each well was rinsed with 2 ml of N2.

Acid extraction and sample preparation. One milliliter of 5% perchloric acid containing 10 μM eserine salicylate (Sigma) was added to each well and the cells were scraped with a rubber policeman, transferred to Eppendorf microtubes, and sonicated. Acid-insoluble material was precipitated by centrifugation in an Eppendorf centrifuge and kept for phospholipid extraction and protein determination. Perchloric acid present in the supernatant was precipitated by the addition of an equimolar concentration of potassium acetate and centrifugation. A 0.8-ml aliquot of the supernatant was dried under vacuum and the dried extracts were kept at −15°C. For analysis the extracts were routinely dissolved in 0.1 ml of 18 mM sodium acetate and filtered through a 0.2-μm Acro LC13 filter (Gelman, Ann Arbor, Mich.), and 20-μl aliquots were injected.

Labeling of hippocampal slices. Male rats (300–400 g) were purchased from Charles River, Wilmington, Massachusetts. The rats were killed by decapitation and the brains removed into ice-cold Krebs–Ringer bicarbonate buffer (KRB) containing (in mM): NaCl, 120; KCl, 3.5; $CaCl_2$, 1.3; $MgSO_4$, 1.2; $NaHCO_3$, 25; and D-glucose, 10. The buffer was gassed for at least 30 min prior to use with a mixture of 95% O_2/5% CO_2. Hippocampi were excised and slices (300 μm thick) were prepared with a McIlwain tissue chopper. Slices (15/tube) were rinsed twice with 4 ml of KRB and incubated in 0.5 ml of KRB containing 5 μCi of [^{3}H]choline (125 nM) for 30 min at 37°C under an atmosphere of 95% O_2/5% CO_2. Following incubation the medium was discarded, slices were rinsed twice with 4 ml of ice-cold KRB, and 1 ml of 5% perchloric acid was added to each tube. Acid extraction then proceeded as described above.

RESULTS AND DISCUSSION

The suitability of the normal phase silica matrix to serve as a stationary phase in HPLC separation of choline metabolites was affirmed in preliminary experiments in which a clear separation of phosphorylated choline metabolites from nonphosphorylated ones was found to be critically dependent on the polarity of the mobile phase. The separation of betaine, acetylcholine, and choline from the phosphorylated choline metabolites was therefore effected using a low-polarity (high acetonitrile concentration) buffer; under these conditions the phosphorylated metabolites were retained. These metabolites were subsequently eluted by a gradient of increasing polarity. In further experiments, the effects of changing ionic strength on the retention times of the various compounds were characterized. It was found that the retention times of the standards, most notably those of acetylcholine and CDP-choline, were sensitive to the ionic strength of the mobile phase. Both acetylcholine and CDP-choline were greatly retarded under conditions of low ionic strength, and this property was

utilized to resolve acetylcholine from choline and CDP-choline from phosphorylcholine. Thus, the introduction of a gradient of increasing polarity and ionic strength allowed the complete separation of the major cellular choline-containing compounds. The chromatography of choline metabolites on a normal phase silica column appears therefore to be of the combined partition/adsorption type.

Figure 1 illustrates the separation of a mixture of six radioisotopically labeled water-soluble choline metabolites by HPLC under the conditions described under Materials and Methods. Retention times of these compounds are summarized in Table 1. All peaks are resolved with baseline or near-baseline resolution. An unidentified peak eluting at the solvent front probably results from degradation of the standards and was found to increase in size upon prolonged storage. Similarly, the relatively high background occurring between the betaine and acetylcholine peaks appears to constitute a degradation product(s) of the glycerophosphocholine standard. We have observed that the chromatographic behavior of choline metabolites may vary slightly among different columns. In these cases, full resolution could be restored by minor adjustments in the gradient's profile.

The ability of this HPLC procedure to separate metabolically labeled choline-containing compounds derived from both a neuronal cell line and hippocampal slices incubated with [^{3}H]choline *in vitro* is demonstrated in Figs. 2 and 3. As shown in Fig. 2, labeling of NG108-15 cells with [^{3}H]choline under the conditions used results in the labeling of two major peaks, exhibiting the retention times of phosphorylcholine (40 min) and glycerophosphocholine (26 min), and a minor peak of [^{3}H]choline. Alkaline phosphatase treatment of the extract resulted in the complete disappearance of the peak eluting at 40 min, and a quantitative transfer of the counts to the position of choline. These findings establish the presence of a phosphomonoester bond in this compound and identify it as phosphorylcholine. The peak eluting at 26 min was not affected by this treatment as would be expected of glycero-

TABLE 1

RETENTION TIMES OF STANDARD RADIOLABELED CHOLINE METABOLITES

Standard	Retention time (min)
[^{3}H]Betaine	10.1 ± 0.4
[^{14}C]Acetylcholine	17.7 ± 0.5
[^{3}H]Choline	22.0 ± 0.0
[^{3}H]Glycerophosphocholine	25.9 ± 0.4
[^{14}C]CDP-choline	30.6 ± 0.8
[^{3}H]Phosphorylcholine	40.1 ± 0.9

Note. Values represent means ± SD of results obtained in seven separate runs.

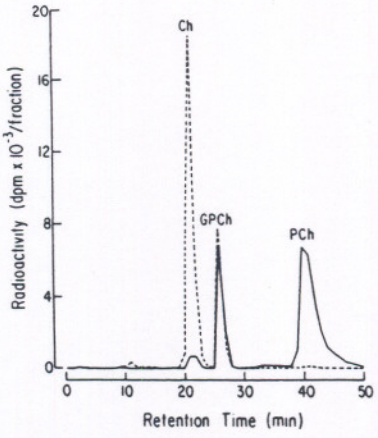

FIG. 2. Effect of alkaline phosphatase on the radiochromatographic profile of acid extracts from [^{3}H]choline-labeled NG108-15 cells. NG108-15 cells were labeled with [^{3}H]choline and extracted as described under Materials and Methods. For alkaline phosphatase digestion, two extracts were dissolved each in 65 μl of buffer containing 0.2 M glycine, 2 mM $MgCl_2$, and 2 mM $ZnSO_4$, pH 10.7. The pH was readjusted to 10.7 with 1 N KOH and the volume adjusted to 130 μl with H_2O. The two extracts were then combined, filtered, and divided into two equal 100-μl portions. 10 μl of a 100 U/ml alkaline phosphatase solution (Sigma) was added to one portion while 10 μl of assay buffer was added to the control tube. Tubes were incubated for 4 h at 37°C, and the reaction was terminated by heating in a boiling water bath for 2 min. Mixtures were then filtered and a 20-μl aliquot of each tube was analyzed by HPLC. (—) Untreated control extract; (---) alkaline phosphatase-treated extract.

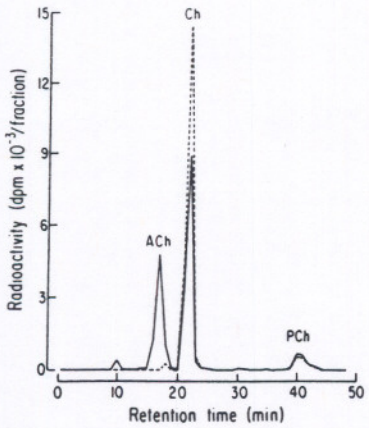

FIG. 3. Effect of acetylcholinesterase on the radiochromatographic profile of acid extracts from [^{3}H]choline-labeled hippocampal slices. Hippocampal slices were labeled with [^{3}H]choline and extracted as described under Materials and Methods. For AChE digestion, an extract was dissolved in 0.1 M sodium phosphate buffer, pH 7.4. The pH was readjusted to 7.5 with 1 N KOH and the extract was filtered and divided into two equal portions of 90 μl. Electric eel AChE (10 μl, 1000 units/ml, Sigma) was added to one tube and 10 μl of buffer was added to the control tube. Tubes were incubated for 120 min at 23°C. Reaction was terminated by the addition of 10 μl of bovine serum albumin (10 mg/ml, Sigma) and precipitation of protein by diluting the mixture with 110 μl of buffer A. Mixtures were cleared of precipitate by filtration and a 20-μl aliquot of each tube was analyzed by HPLC. (—) Untreated control extract; (---) acetylcholinesterase-treated extract.

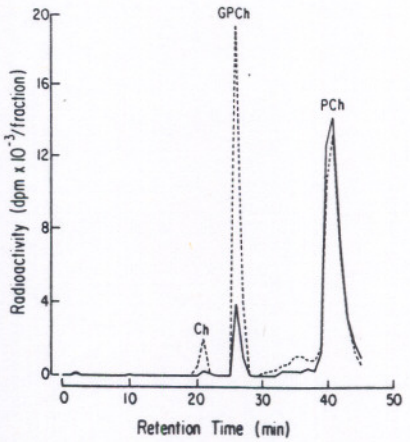

FIG. 4. Stimulation of [^{3}H]glycerophosphocholine labeling by the Ca^{2+} ionophore A23187. Cells were subcultured and grown essentially as described under Materials and Methods, with minor modifications. The cells were labeled by incubation with 5 μCi/well of [^{3}H]choline in N2 medium without choline, methionine, tryptophan, serine and linoleic acid (N2(−)) for 1 hour. After incubation the cells were rinsed with N2(−) and chased for 8 hours in normal growth medium (choline concentration of 30 μM), in the absence or presence of 5 μM A23187 (Sigma). This was followed by extraction and HPLC analysis as described in Materials and Methods. (—) Untreated control culture; (---) A23187-treated culture.

phosphocholine. However, no further attempt was made to establish the identity of this peak.

In marked contrast to the distribution of labeled choline metabolites in NG108-15 cells, incubation of hippocampal slices with [^{3}H]-choline (Fig. 3) resulted primarily in the labeling of the free choline pool and of a radioactive peak exhibiting the retention time of acetylcholine (18 min). Treatment of these extracts with acetylcholinesterase, an enzyme that specifically hydrolyzes the acetylcholine ester bond, caused the virtual disappearance of the peak eluting at 18 min; the lost counts were quantitatively recovered at the position of choline, identifying this peak as acetylcholine.

The HPLC method presented here has been applied in a variety of studies concerned with acetylcholine and phosphatidylcholine me-

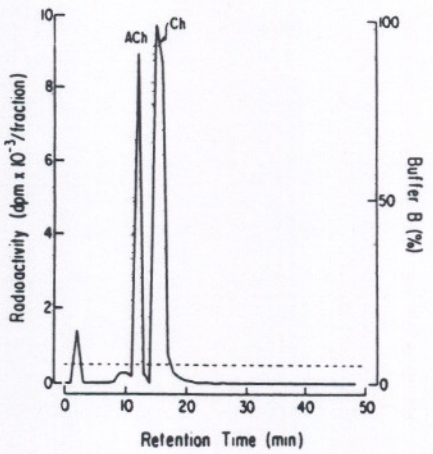

FIG. 5. Isocratic separation of [^{14}C]acetylcholine and [^{3}H]choline standards. A mix of [^{14}C]acetylcholine (ACh) and [^{3}H]choline (Ch) standards was separated utilizing a mixture of 95% buffer A and 5% buffer B. Other details of the chromatographic conditions, including buffer composition are described in the legend to Fig. 1 and under Materials and Methods.

tabolism in which [^{3}H]choline was utilized as a precursor. In one of these studies, for example, the effects of increasing the intracellular concentration of Ca^{2+} on phosphatidylcholine metabolism were investigated. To illustrate the utility of the present HPLC method, some of the results obtained in this study are shown in Fig. 4. In this experiment, NG108-15 cells were pulse labeled with [^{3}H]choline for 1 h and then chased for 8 h in the presence of A23187, a Ca^{2+} ionophore. HPLC analysis of the acid-soluble choline metabolites revealed a marked increase in the levels of two radioactive peaks exhibiting the retention times of [^{3}H]glycerophosphocholine and [^{3}H]choline, suggesting that A23187 stimulated phosphatidylcholine catabolism in these cells. This finding is consistent with previous reports on the Ca^{2+} dependence of phospholipase A_2 and glycerophosphocholine phosphodiesterase (20,21), and shows that these enzymes can probably be activated in intact cells by elevating intracellular Ca^{2+} concentrations.

Figure 5 demonstrates the possible utilization of isocratic elution conditions, selected on the basis of standard gradient conditions, to separate a partial set of the choline metabolites. In this case, choline and acetylcholine standards were separated from each other within 25 min with a mobile phase consisting of 95% buffer A/5% buffer B. Isocratic elution conditions that will allow the rapid separation of any pair of choline metabolites may be similarly and easily developed from the gradient conditions described in this presentation. Such isocratic conditions could be useful in the separation of products from substrates in assays of various enzymes involved in choline metabolism.

CONCLUSIONS

We have presented a new method for separating choline metabolites by HPLC. This method may be useful in studies of acetylcholine and phosphatidylcholine metabolism, and of the possible interrelationships of these compounds in cholinergic cells.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institute of Mental Health, the Department of Defense, and the Centre for Brain Sciences and Metabolism Charitable Trust. M.L. is a recipient of the Myron A. Bantrell Post-doctoral Fellowship.

Note added in proof. We have recently found that the mobile phase can be made totally volatile by substituting ammonium acetate for sodium acetate (at the same concentration) without affecting retention times. This modification facilitates the use of the present HPLC method in preparative applications.

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