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Quantitation of Choline and Its Metabolites in Tissues and Foods by Liquid Chromatography/Electrospray Ionization-Isotope Dilution Mass Spectrometry

Hasan Koc,[†] Mei-Heng Mar,[‡] Asoka Ranasinghe,[†] James A. Swenberg,[†] and Steven H. Zeisel*[,‡]

Department of Environmental Sciences and Engineering and Department of Nutrition, University of North Carolina, Chapel Hill, North Carolina 27599

Choline is important for normal membrane function, acetylcholine synthesis, lipid transport, and methyl metabolism. The U.S. National Academy of Sciences recently set requirements for choline in the human diet. In tissues and foods, there are multiple choline compounds that contribute to choline content. Betaine, a derivative of choline, is also important because of its role in donation of methyl groups to homocysteine to form methionine. Radioisotopic, high-pressure liquid chromatography, and gas chromatography/isotope dilution mass spectrometry (GC/IDMS) methods are available for measurement of choline. However, these existing methods are cumbersome and time-consuming, and none measures all of the compounds of interest. In this study, we describe a new method for quantitation of choline, betaine, acetylcholine, glycerophosphocholine, cytidine diphosphocholine, phosphocholine, phosphatidylcholine, and sphingomyelin in liver, plasma, various foods, and brain using liquid chromatography/electrospray ionization-isotope dilution mass spectrometry (LC/ESI-IDMS). Choline compounds were extracted by and partitioned into organic and aqueous phases using methanol and chloroform and analyzed directly by LC/ESI-IDMS without the need for isolation and derivatization of each compound separately as was required by the GC/IDMS method. The new LC/ESI-IDMS method was validated using the existing published GC/IDMS method.

Choline (Cho), a component of many foods, is part of several major phospholipids (including phosphatidylcholine (PtdCho) and sphingomyelin (SM)) that are critical for normal membrane structure and function.[1] Choline is also a precursor of betaine, used by the kidney to maintain water balance[2] and by the liver as a source of methyl groups for methionine formation.[3] Finally, choline is used to produce the important neurotransmitter acetylcholine.[4] Cells die by apoptosis when deprived of choline,[5−7] and humans eating a choline-deficient diet develop liver dysfunction.[8] This led the U.S. National Academy of Sciences to recommend dietary intake levels for choline in humans.[9] There are multiple forms of choline in biological tissues, the major compounds being Cho, acetylcholine (AcCho), phosphocholine (PCho), glycerophosphocholine (GPCho), cytidine diphosphocholine (CDP-Cho), PtdCho, and SM.[1] Structures of these choline compounds are given in Figure 1. Betaine, as discussed earlier, is derived from choline. Methods already are available for quantitation of many of the above compounds. Cho and AcCho can be measured using a radioisotopic procedure,[10] but this difficult assay has been largely supplanted by a procedure that uses high-pressure liquid chromatography (HPLC), an enzyme reactor, and electrochemical detection after a simple sample pretreatment.[11,12] Alternative methods include using HPLC and fluorometric detection[13] and HPLC with continuous-flow fast atom bombardment mass spectrometry.[14] These methods are accurate, reliable, and inexpensive but are only suitable for measurement of Cho and AcCho and they do not permit the use of internal standards. A gas chromatography/isotope dilution mass spectrometry (GC/IDMS) method requires much more expensive equipment but has the advantage that it has been adapted to measure other choline compounds. The original method[15] has been enhanced by a preseparation of the various water-soluble choline metabolites using HPLC.[16] This procedure permits analysis of Cho, AcCho, PCho, and GPCho in

* Corresponding author. E-mail: steven_zeisel@unc.edu. Tel: 919-966-7218. Fax: 919-966-7216.

[†] Department of Environmental Sciences and Engineering.

[‡] Department of Nutrition.

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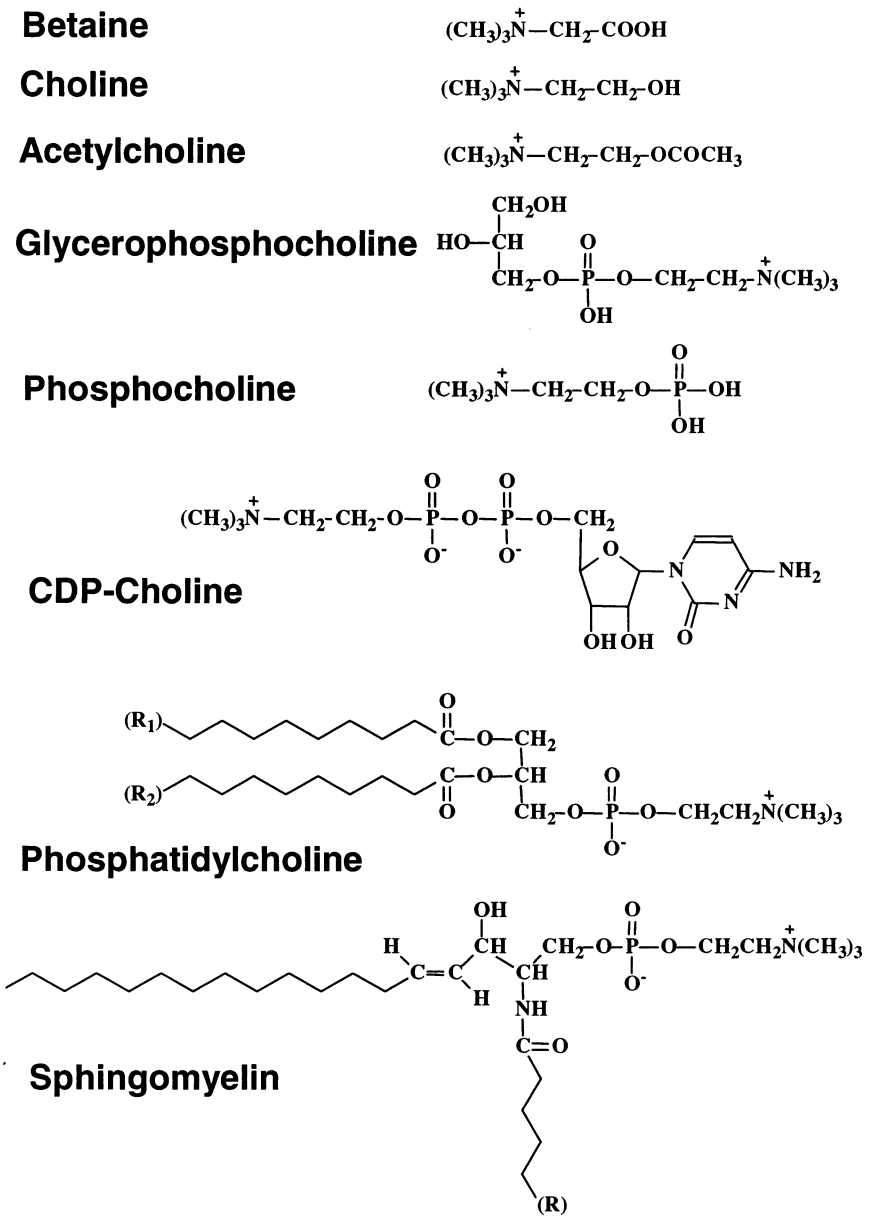

Figure 1. Structures of choline compounds studied.

a single sample. It is a demanding procedure since it requires that the separate HPLC peaks be collected and the metabolite in question be converted to Cho and then be derivatized prior to the GC/IDMS analysis. It does permit the inclusion of an isotopically labeled internal standard for each metabolite, thereby ensuring greater accuracy and reliability when widely divergent matrixes are to be analyzed (e.g., tissues and foods). The GC/IDMS method, however, could not measure betaine. Betaine can be isolated using HPLC and derivatized with 4′-bromophenacyl triflate so that it can be detected and quantified using UV absorbance.[17] Several researchers have developed liquid chromatography/mass spectrometry (LC/MS) methods for quantitation of Cho and its metabolites. An LC/ESI-MS method was developed for quantitation of AcCho in cell cultures.[18] Recently, Zhu et al.[19] developed a reversed-phase ion-pairing liquid chromatography/mass spectrometry method for quantitation of Cho, AcCho, carnitine, and acetylcarnitine in rat brain. There is still no LC/MS method for quantitation of betaine, PCho, CDP-Cho, and GPCho. In this report, we describe a liquid chromatography/electrospray ionization-isotope dilution mass spectrometry (LC/ESI-IDMS) method that can quantitate betaine, AcCho, Cho, GPCho, CDP-Cho, PCho, PtdCho, and SM in various tissues and foods. The new LC/ESI-IDMS method greatly reduces the number of steps in the analysis of the various choline compounds because it requires no prior fractionation by HPLC and no derivatization. Time required for analysis of a batch of samples ($n = 25$) by the

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GC/MS method has now been reduced from approximately 10 to 2 days.

EXPERIMENTAL SECTION

Reagents. All reagents were obtained from Fisher Chemicals (Pittsburgh, PA) unless otherwise noted. Betaine, acetylcholine chloride, choline chloride, glycerophosphocholine, cytidine diphosphocholine, and phosphocholine were from Sigma Chemicals (St. Louis, MO). Choline-[*N*,*N*,*N*-trimethyl-d_9] bromide, acetylcholine-[*N*,*N*,*N*-trimethyl-d_9] bromide, betaine-[*N*,*N*,*N*-trimethyl-d_9] hydrochloride, and phosphocholine-[*N*,*N*,*N*-trimethyl-d_9] chloride were purchased from CNS isotopes (Quebec, Canada). 1,2-Dipalmitoyl-*sn*-3-glycero-phosphocholine-[*N*,*N*,*N*-trimethyl-d_9] was purchased from Avanti Polar Lipids (Alabaster, AL). Glycerophosphocholine-[*N*,*N*,*N*-trimethyl-d_9] and sphingomyelin [*N*-methyl-d_3] were synthesized as described below. All solvents used were of HPLC grade.

Instrumentation. The LC/MS system consisted of an LCQ-^{DECA} (ThermoQuest, San Jose, CA) quadrupole ion trap mass spectrometer equipped with an API2 electrospray ionization (ESI) source, a Surveyor HPLC system (ThermoQuest), and a Surveyor autosampler (ThermoQuest) with a refrigerated sample tray.

Synthesis of Glycerophosphocholine-[*N*,*N*,*N*-trimethyl-d_9]. Glycerophosphocholine-[*N*,*N*,*N*-trimethyl-d_9] was synthesized from 1,2-dipalmitoyl-*sn*-3-glycerophosphocholine-[*N*,*N*,*N*-trimethyl-d_9] by selective mild hydrolysis as described previously.[16] Briefly, 10 μmol of 1,2-dipalmitoyl-*sn*-3-glycerophosphocholine-[*N*,*N*,*N*-trimethyl-d_9] was hydrolyzed in 1 mL of chloroform/methanol (1:4) mixture with 100 μL of 1.2 N NaOH in methanol/water (1:1) at 37 °C for 10 min. After incubation, the reaction mixture was neutralized with 150 μL of 1 N acetic acid. The product was extracted with a mixture of 2 mL of chloroform/methanol (9:1), 1 mL of isobutanol, and 2 mL of water. The lower phase was re-extracted twice with 1 mL of methanol/water (1:2). Aqueous phases were combined and dried using a vacuum centrifuge (Speed-Vac, Savant Instruments, Farmingdale, NY). Glycerophosphocholine-[*N*,*N*,*N*-trimethyl-d_9] was purified by an HPLC method described previously.[16]

Synthesis of Sphingomyelin-[*N*-methyl-d_3]. Sphingomyelin-[*N*-methyl-d_3] was prepared by the procedure of Stoffel.[20] Briefly, 1 mmol of sphingomyelin and 6 mmol of 1,4-diazabicyclo(2,2,2)-octane dissolved in 50 mL of dimethyl formamide were refluxed under a stream of nitrogen for 6 h. After cooling, the solution was poured on 20 mL of ice-cold 1 N HCl and the reaction product was extracted with five 50-mL portions of chloroform. The combined extracts were dried by vacuum centrifugation (Speed-Vac), and 200 mg (0.275 mmol) of the resulting ceramide-1-phosphoryl-*N*,*N*-dimethylethanolamine was mixed with 30 μL of (0.35 mmol) cyclohexylamine, and 90 μL (0.28 mmol) of iodomethane-d_3 in 5 mL of dry methanol in a tightly sealed Teflon-lined screw-cap tube that was stored at room temperature in the dark for ~18 h. At the end of the reaction, the solvent was evaporated under vacuum, the residue was dissolved in chloroform, and the chloroform solution was washed twice with 5% $Na_2S_2O_3$, 2 N HCl, and water. The organic phase was dried by vacuum centrifugation (Speed-Vac). The products were purified on a Bond Elut aminopropyl column using the procedure described in the Sample Preparation section.

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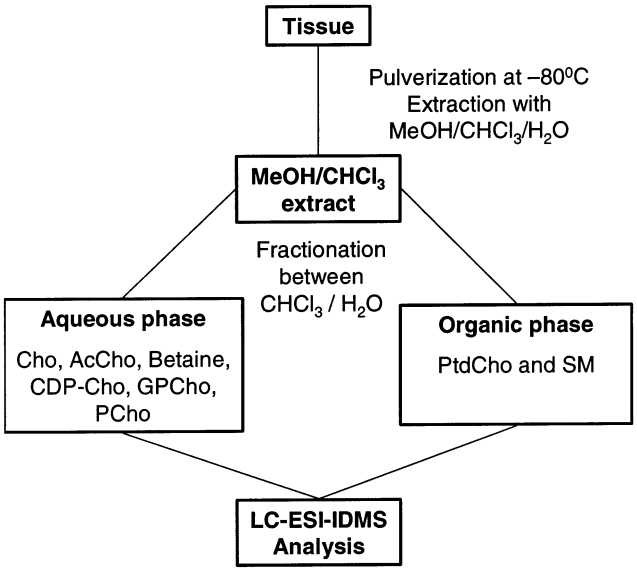

Figure 2. Scheme for analysis of tissue and food samples for choline compounds.

Sample Preparation. A variety of samples were processed for analysis of aqueous choline compounds. These samples included mouse liver, rat fetal brain, plasma, cooked beef and chicken, raw carrot, and cabbage. A schematic overview of the method is given in Figure 2. All the samples were either analyzed immediately or stored at −80 °C until the time of analysis. Choline compounds, with the exception of AcCho, were extracted from tissues and foods using the method of Bligh and Dyer.[21] Briefly, liver and food samples were frozen at −80 °C and pulverized. A known amount (e.g., 100 mg) was taken from each sample and spiked with deuterium-labeled internal standards of all the analytes (PCh-d_9 was used for analyses of CDP-Cho). For a 100-mg sample of tissue or food, 400 μL of methanol/chloroform (2:1, v/v) was added, and the resultant mixture was vortexed vigorously and left at −20 °C overnight. At the end of the extraction with methanol/chloroform, tissue samples were centrifuged at 1500*g* for 5 min. The supernatants were transferred to new tubes, and the residues were re-extracted with 250 μL of methanol/chloroform/water (2:1:0.8, v/v). The supernatants from both extractions were combined. To the resulting solution, first 100 μL of chloroform and then 100 μL of water were added to form two phases. After centrifugation, the entire aqueous phase (which contained betaine, AcCho, Cho, PCho, GPCho, and CDP-Cho) was separated from the chloroform phase (which contained the phospholipids PtdCho and SM). The aqueous phase was dried by vacuum centrifugation (Speed-Vac) and redissolved in 20 μL of water. To the aqueous solution, 800 μL of methanol was added. This resulted in precipitation of some unwanted compounds from the solution. These insoluble compounds, mostly proteins, were removed by centrifugation. A portion (10 μL) of this final supernatant was analyzed by LC/ESI-IDMS. PtdCho and SM, which were in the bottom organic phase, were dried, resuspended, and separated from other lipids using a Bond Elut aminopropyl column (Varian Analytical Instruments, Walnut Creek, CA) before the LC/ESI-IDMS analysis. The column was conditioned with 5 mL of hexane.

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Samples were constituted in 0.5 mL of chloroform and applied to the column. After washing the column with 4 mL of chloroform/2-propanol (v/v, 2:1) and 4 mL of 2% acetic acid in ethyl ether, PtdCho and SM were eluted with 5 mL of methanol. This solution was further diluted based on the expected contents of the sample to fall within the linear range of the assay and analyzed by LC/ESI-IDMS.

Sample Preparation for AcCho. Because most tissues and foods contain no appreciable amounts of this choline ester, AcCho was analyzed only in two cholinergic cell lines treated so that they increased synthesis of acetylcholine (murine SN56T17 cells and human LA-N-2 neuroblastoma cells; kindly provided by Dr. J. K. Blusztajn).

Murine SN56T17 cells were plated on 35-mm dishes and grown in Dulbeccos Modified Eagle Media (DMEM; Life Technologies, Inc., Rockville, MD) containing 10% FBS for 24 h. Then the medium was replaced and the cells were grown in the presence or absence of 1 mM dibutyryl cAMP for 48 h. Human LA-N-2 neuroblastoma cells were plated on 35-mm dishes and grown in L-15 (Life Technologies, Inc.) medium containing 10% FBS (Life Technologies, Inc.) for 24 h. Then the medium was replaced and the cells were grown in the presence or absence of 20 ng/mL ciliary neurotrophic factor for 48 h. At the end of the incubation, the medium was removed and the cells were incubated for 1 h at 37 °C in a physiological salt solution (pH 7.4): NaCl 135 mM, KCl 5 mM, $CaCl_2$ 1 mM, $MgCl_2$ 0.75 mM, glucose 10 mM, Hepes 10 mM, supplemented with 10 μM choline and 15 μM neostigmine. After incubation, the cells were washed once with ice-cold, choline-free physiological salt solution supplemented with 15 μM neostigmine. The cells were scraped into methanol/1 M formic acid (10:1 v/v) and centrifuged at 1500*g* for 10 min and the protein pellets assayed for protein,[22] whereas the supernatants were extracted using chloroform/water (2:1, v/v). The samples were mixed by vortex and centrifuged for 10 min at 3000*g*. The aqueous phase was collected and dried under vacuum. The dried sample was reconstituted in 0.2 mL of water and analyzed by LC/ESI-IDMS.

LC/ESI-IDMS Analysis. Aqueous Phase. Samples were maintained at 10 °C while they were in the autosampler tray, and 10 μL from each sample was injected. Guard and analytical columns were Alltech Adsorbosphere Silica (4.6 × 25 mm, 5 μm) and Alltech Solvent Miser Silica (2.1 × 150 mm, 5 μm), respectively. An acetonitrile/water/ethanol/1 M ammonium acetate/glacial acetic acid (800/127/68/3/2, v/v; solvent A) and acetonitrile/water/ethanol/1 M ammonium acetate/glacial acetic acid (500/500/85/27/18; solvent B) gradient was used for baseline separation of all the compounds. The gradient was 0% B at 0.4 mL/min for 3 min to 40% B in 7 min, to 45% B and 0.3 mL/min in 4 min, to 60% B in 4 min, to 100% B and 0.5 mL/min in 1 min, at 100 % B for 8 min, to 0 % B in 1 min, to 0.4 mL/min in 2 min. Both the guard and the analytical columns were heated to 40 °C. Positive ESI parameters were tuned for best sensitivity under HPLC conditions employed using the automatic tuning procedure recommended by the manufacturer. Nitrogen was used as both sheath and auxiliary gases that were set at 80 and 20 arbitrary units, respectively. The spray voltage for ESI was 5 kV. The heated capillary of the ESI source was kept at 350 °C during analysis. Already positively charged molecular ions of all analytes and their d_9-labeled internal standards were monitored in selected ion monitoring (SIM) mode for mass spectrometric detection. Mass spectrometric detection in SIM mode detection was done in two segments. In segment 1 (5−13 min), betaine, betaine-d_9, AcCho, AcCho -d_9, Cho, and Cho-d_9 were detected; in segment 2 (13−26 min), GPCho, GPCho -d_9, CDP-Cho, PCho, and PCho-d_9 were detected. Structures of the analytes studied are given in Figure 1. To minimize source contamination and the possibility of signal suppression due to the presence of matrix components, a six-port valve (Valco) was used to divert the LC effluent to waste before and after a selected retention time window (5−26 min) in which analytes of interest eluted. Data collection and analysis was performed by XCalibur software running on Windows NT 4.0.

Organic Phase. The LC/ESI-IDMS analysis of the organic phase was done the same way as the aqueous phase with the exception of the HPLC gradient and the mode of mass spectrometric detection. The gradient used for separation of the phospholipids PtdCho and SM was 0% B at 0.4 mL/min for 3 min to 20% B in 7 min, to 60% B and 0.3 mL/min in 4 min, to 100% B in 2 min, to 100% B and 0.5 mL/min in 1 min, at 100% B for 3 min, to 0% B in 1 min, to 0.4 mL/min in 1 min. SIM of positively charged molecular ions, as in the case of aqueous choline compounds, was not suitable for the phospholipids in the organic phase since PtdCho and SM are groups of compounds with different long-chain hydrophobic moieties. However, both PtdCho and SM conveniently contain a PCho moiety. As we expected, in-source fragmentation of PtdCho and SM produced PCho as a common characteristic ion. This phenomenon was exploited in the SIM detection. As a result, both PtdCho and SM were detected simply by monitoring a single m/z (m/z 184). The m/z's monitored for deuterium-labeled internal standards of PtdCho (PtdCho-d_9) and SM (SM-d_3) were m/z 193 and 187, respectively. All the m/z's were monitored simultaneously. LC effluent was allowed into the mass spectrophotometer between 4 and 14 min. It was directed to waste to minimize source contamination and signal suppression during the other remaining times at the beginning and the end of the run.

In addition to quality control samples, blanks were routinely analyzed to avoid any carryover that might occur especially after analysis of samples with high levels of analytes.

RESULTS AND DISCUSSION

We developed a new method for quantitation of choline compounds using on-line liquid chromatographic separation and detection by electrospray ionization-isotope dilution mass spectrometry. The most obvious strength of this method is the simplicity of its sample preparation procedure that eliminated the complex HPLC fraction collection and derivatization procedures as required by the existing GC/MS procedure[16] with the associated reduction in human labor and total analysis time. After extraction of the choline compounds from tissues, ~10 days was needed for analysis of a batch of samples (~25 samples) using the previously reported GC/IDMS method.[16] The same number of samples could now be analyzed with a fully automated LC/MS system in ~1 day using the newly developed LC/ESI-IDMS method. Other potential benefits of our new procedure include decreased possibility of introduction of human errors and artifacts

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because fewer steps are required in the sample preparation procedure, the excellent specificity provided by the mass spectrometric detection, and finally the increased reliability of quantitation through the use of deuterium-labeled internal standards (not possible in methods that do not use mass spectrometric detection[11,12,23]). The choline compounds were isolated as aqueous soluble (betaine, Cho, AcCho, GPCho, CDP-Cho, PCho) and organic soluble (PtdCho, SM) phases that were analyzed by LC/ESI-IDMS in two separate injections (Figure 2) because different HPLC gradients, which consisted of the same solvents, were required for separation.

Although mass spectrometers can detect and identify mixtures based on their mass differences, on-line HPLC separation plays a critical role in analysis of biological matrixes by mass spectrometry. It adds to the specificity of the method by adding another dimension of purification and reduces the possibility of interferences and signal suppression due to matrix components present in amounts that are much larger than the analytes of interest. Since the choline compounds studied were already charged in the solution due to the presence of quaternary ammonium groups, they were very well suited to detection by electrospray ionization−mass spectrometry without any modification. However, this very nature of the compounds also made difficult their separation by reversed-phase chromatography, the most popular separation technique for on-line LC/MS applications. Separations that we obtained on reversed-phase columns, even with ion-pairing reagents, were not satisfactory (data not shown). Previously, we achieved excellent separation of aqueous choline compounds using normal-phase HPLC[16] and we now modified these conditions to optimize them for ESI-MS. Mainly, potassium phosphate was eliminated from the mobile phase and a column with a smaller internal diameter was used. These modifications in the conditions did not result in any significant deterioration of separation, with baseline separation of all compounds of interest.

Contamination of the electrospray source and the mass spectrometer was a problem when complex matrixes were analyzed, and this was reduced by directing the HPLC effluent to the waste instead of to the mass spectrometer at the beginning and the end of the analysis where no analytes eluted. In addition, only ~1/3 of the HPLC flow was allowed to enter the ESI source by postcolumn splitting of the flow with a Valco T-connector. Postcolumn splitting of the flow did not result in a significant loss in the sensitivity.

Method Validation. Calibration curve standards were prepared by mixing 20 nmol of deuterium-labeled internal standards with varying amounts of analytes. The calibration curves were constructed by relating the varying amounts of each analyte to their relative response factors (RRFs) as determined by the ratio of the peak area of the analyte to that of the corresponding deuterium-labeled internal standard. The calibration curves, working ranges, and correlation coefficients for the choline compounds studied are given in Figure 3. Responses for all the analytes were linear over the working range with correlation coefficients ranging between 0.9942 and 0.9992.

Recovery experiments were conducted to evaluate accuracy of the quantitation. For this purpose, triplicate samples of pooled rat liver were spiked with known amounts of authentic standards. The spiked rat liver samples were then processed the same way as the other samples and analyzed by LC/ESI-IDMS. Excellent recovery values were obtained (100.9 ± 3.7% for betaine; 91.6 ± 4.9% for Cho; 104 ± 3.8% for AcCho; 88.0 ± 7.3% for GPCho; 101.7 ± 1.3% for CDP-Cho; 85.7 ± 4.0% for PCho; 99.6 ± 5.1% for PtdCho; and 104.8 ± 4.4% for SM).

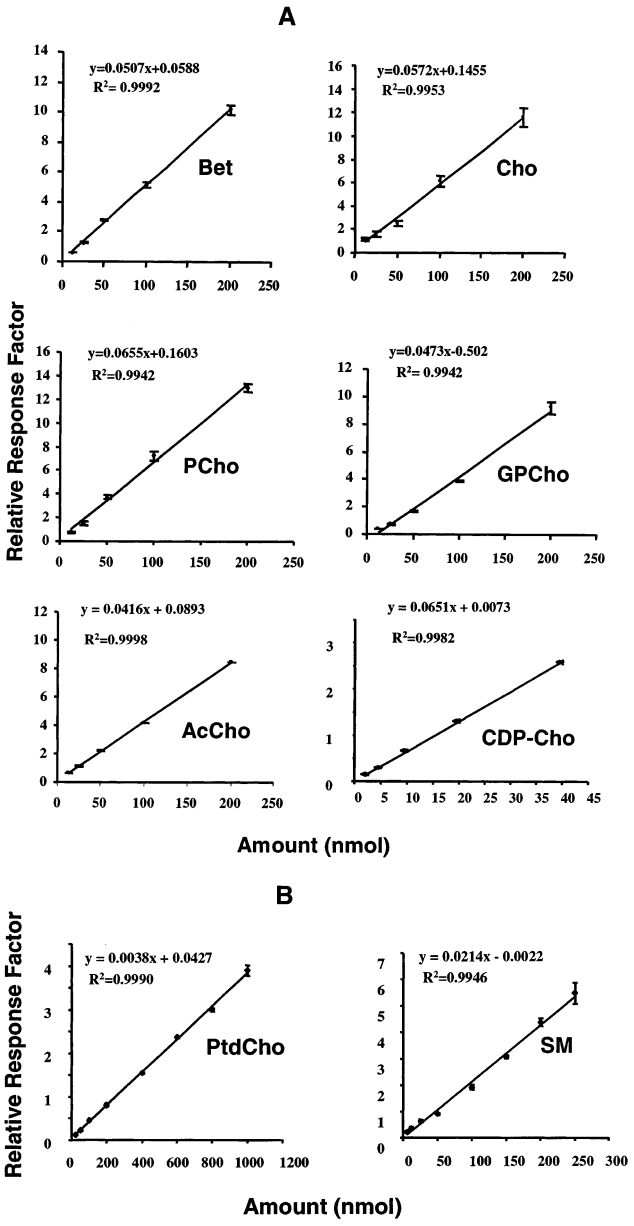

Figure 3. (A) Calibration curves for betaine (Bet), choline (Cho), phosphocholine (PCho), glycerophosphocholine (GPCho), acetylcholine (AcCho), and CDP-choline (CDP-Cho). (B) Calibration curves for phosphatidylcholine (PtdCho) and sphingomyelin (SM). Authentic standards of choline compounds were analyzed using on-line liquid chromatographic separation and detection by electrospray ionization-isotope dilution mass spectrometry as described in the Experimental Section. Calibration curve standards were prepared by mixing 20 nmol of deuterium-labeled internal standards with varying amounts of analytes. The calibration curves were constructed by relating the varying amounts of each analyte to their RRFs as determined by the ratio of the peak area of the analyte to that of the corresponding deuterium-labeled internal standard.

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Table 1. Comparison of Concentration Values Obtained from the Same Samples Using the Newly Developed LC/ESI-IDMS Method and Previously Available Methods[a]

		concentration ± SD (nmol/g)					
sample	method	Bet	Cho	GPCho	PCho	PtdCho	SM
mouse liver	LC/MS	494 ± 16	119 ± 21	146 ± 08	2030 ± 281	20647 ± 372	1834 ± 106
	old	381 ± 19	138 ± 22	111 ± 12	1862 ± 156	19388 ± 1011	1804 ± 178
rat liver	LC/MS	555 ± 31	101 ± 09	155 ± 03	1403 ± 05	19513 ± 201	1635 ± 106
	old	488 ± 26	75 ± 02	124 ± 19	1403 ± 71	21732 ± 1273	1716 ± 29
rat brain	LC/MS	181 ± 07	231 ± 36	526 ± 34	2907 ± 13	11267 ± 68	933 ± 81
	old	161 ± 37	192 ± 15	587 ± 37	3192 ± 323	10590 ± 489	951 ± 43
chicken (cooked)	LC/MS	361 ± 18	285 ± 67	277 ± 07	262 ± 12	7774 ± 468	1132 ± 49
	old	321 ± 10	231 ± 25	287 ± 25	265 ± 47	7517 ± 287	1333 ± 72
beef (cooked)	LC/MS	1338 ± 214	196 ± 16	216 ± 12	89 ± 17	5816 ± 314	611 ± 17
	old	906 ± 07	124 ± 17	262 ± 46	169 ± 52	5653 ± 59	644 ± 126
carrot (raw)	LC/MS	19 ± 02	357 ± 41	nd	106 ± 07	436 ± 12	nd
	old	21 ± 13	396 ± 65	nd	108 ± 06	200 ± 31	nd
cabbage (raw)	LC/MS	22 ± 07	543 ± 31	201 ± 12	123 ± 29	278 ± 14	nd
	old	38 ± 10	579 ± 30	172 ± 19	167 ± 35	130 ± 27	nd

[a] nd, not detected. All the samples were analyzed in triplicates ($n = 3$).

Specificity of the method was tested by spiking one of the calibration curve standards mixture with high levels (10 times excess) of carnitine and creatinine; biochemically related compounds that could typically be present in the biological matrix in large amounts. The presence of either of these compounds at the indicated amounts did not affect the results of quantitation (data not shown).

Intra- and interassay reproducibilities of the method were assessed by repeating the analysis of a sample 10 times in a single determination and by repeating analysis of this sample in five separate assays on different days. Coefficients of intra-assay variation for measurements were 2.4% for betaine, 2.1% for AcCho, 8.5% for Cho, 7.0% for GPCho, 3.2% for CDP-Cho, 5.4% for PCho, 1.6% for PtdCho, and 3.5% for SM. Coefficients of interassay variation for measurements were 2.8% for betaine, 3.0% for AcCho, 6.9% for Cho, 6.2% for GPCho, 4.5% for CDP-Cho, 1.2% for PCho, 1.2% for PtdCho, and 1.9% for SM. To monitor reproducibility of quantitation, in analyses of tissue or foods we routinely included a standard mixture and a pooled liver extract.

Detection limits for compounds ranged between 1 and 40 pmol using pure standards injected on column, with betaine detection being the most sensitive and GPCho detection being the least sensitive. Quantitation of all the choline compounds was possible consuming only 1/80 of the sample. Due to the relatively high concentrations present in the samples analyzed, the sensitivity of the mass spectrometer was not an issue. Mass spectrometers with quadrupole analyzers can provide much better sensitivity if such a need for sensitivity arises. The preliminary studies in the development of this method were actually done using such an instrument.

Concentrations of the choline compounds determined using our new method were validated using previously developed methods. For this purpose, the samples representing different biological matrixes were analyzed using both the new LC/ESI-IDMS method and the previously available methods, which were the HPLC method for betaine,[17] the phosphorus assay method for PtdCho and SM,[24] and the GC/IDMS method[16] for the rest of the analytes. The comparison of the two data sets is shown in Table 1. The values generated by the new assay were highly comparable to those from the previously used methods. We suggest that differences are likely due to inaccuracies introduced by the older methods during the multiple processing steps required. There is very little data on concentrations of the choline compounds in the literature. The data generated in our study are also similar to those limited values that can be found in the literature.[16,25−27]

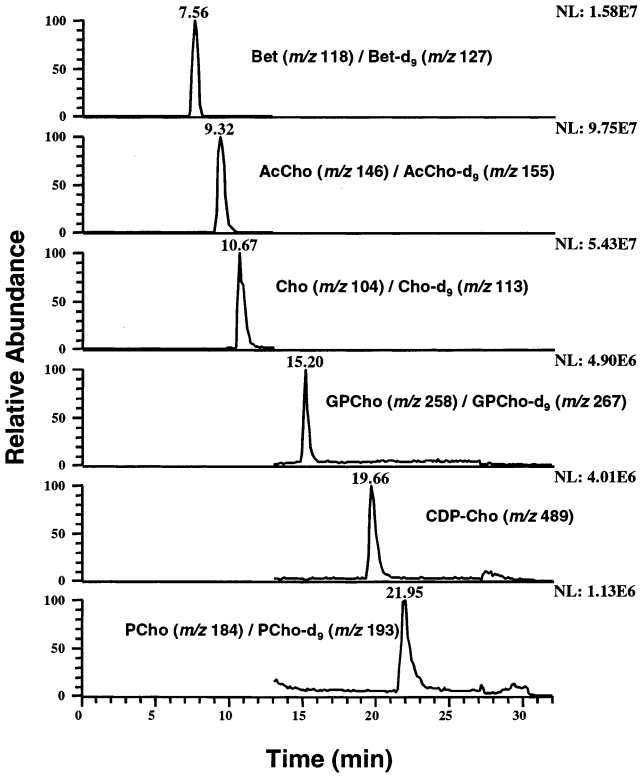

Figure 4. Selected ion chromatograms Bet, AcCho, Cho, (GPCho), CDP-choline CDP-Cho, and PCho. Authentic standards of choline compounds were analyzed using on-line liquid chromatographic separation and detection by electrospray ionization-isotope dilution mass spectrometry as described in the Experimental Section.

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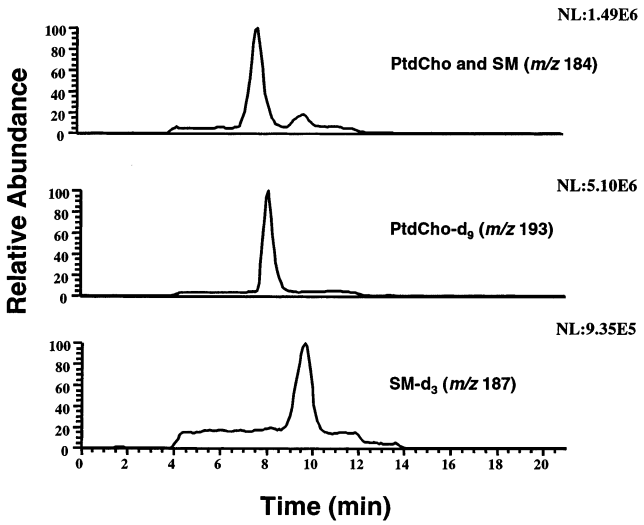

Figure 5. Selected ion chromatograms of PtdCho and SM from a cooked beef extract. Tissue was extracted and choline compounds were analyzed using on-line liquid chromatographic separation and detection by electrospray ionization-isotope dilution mass spectrometry as described in the Experimental Section.

LC/ESI-IDMS Analysis of Food and Tissue Samples. We used the newly developed method for analysis of samples from liver, brain, and foods including cooked beef and chicken, carrots, and cabbage. The same sample preparation procedure was used for all matrixes. The amount of the extraction medium was adjusted proportionally based on the amount of the sample. We also applied this method to the analysis of cultured cells, human plasma, and urine (data not shown). Typical sets of SIM traces for aqueous and organic soluble choline compounds are shown in Figures 4 and 5, respectively. Measured levels of choline compounds in cooked and raw vegetables were quite different, differing especially in Cho and PtdCho concentrations. We discovered that when mincing plant tissues (vegetables), phospholipase D was activated, resulting in the conversion of PtdCho to phosphatidic acid and Cho (we assessed this using thin-layer chromatography). This enzyme activity can be inhibited by boiling the raw vegetable in water before mincing (at least 3 min; our data not shown) or by homogenizing the fresh vegetable in hot (82 °C) 2-propanol.[28]

AcCho was not detected in most foods and tissues because it is rapidly hydrolyzed by cholinesterases unless special collection techniques are used.[29] Therefore, we used cholinergic cells in culture before and after treatments that increased synthesis of acetylcholine to test our assay. As expected, murine SN56717 cells after treatment with dibutyryl cAMP had higher AcCho concentrations (1.14 ± 0.12 nmol/mg protein) than they did at baseline (0.28 ± 0.12 nmol/mg protein). Similarly, LAN-2 cells treated with ciliary neurotrophic factor had higher AcCho concentrations (14.9 ± 0.3 nmol/mg protein) than they did at baseline (10.0 ± 0.06 nmol/mg protein).

In conclusion, we have developed an LC/ESI-IDMS method that allows one to quantitate eight choline compounds. The new method reduced the analysis time significantly and eliminated the extremely laborious sample preparation procedure required by its GC counterpart, therefore reducing the possibilities of introducing human errors and artifacts. This method has the potential for further automation and improvements that would allow one to reduce analysis time even further by, for example, using automated column-swapping techniques.

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