Quantitative gas–liquid chromatographic analysis of ethanolamine, monomethyl ethanolamine, and dimethyl ethanolamine from lipids

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ABSTRACT A method for the separation and quantitative estimation of ethanolamine, dimethyl ethanolamine, and monomethyl ethanolamine derived from lipids is described. After acid hydrolysis of the lipids, the bases are extracted from the neutralized hydrolysate with *t*-butanol and separated by gas–liquid chromatography.

KEY WORDS dimethyl ethanolamine · ethanolamine · monomethyl ethanolamine · gas–liquid chromatography · phospholipids · quantitative assay

PHOSPHATIDYL CHOLINE can be formed by successive methylations of phosphatidyl ethanolamine (1, 2). Methylated ethanolamines have been detected in fungi (3), yeast (4), bacteria (5), and animal tissues (1, 2). In mammals there are variations in the activity of this pathway in various organs (6, 7) and between the sexes (6). A simple, rapid, and quantitative analytical method for EA, DMEA, and MMEA would be very useful. A method employing lipid extraction, acid hydrolysis, extraction of the hydrolysis mixture after neutralization, and GLC analysis without derivative formation is described in this report.

MATERIALS AND METHODS

Sources of Phospholipids

The brain, heart, psoas muscles, and liver of a young male white rat were extracted with chloroform–methanol as described by Bligh and Dyer (8). *Hemophilus parainfluenzae* was grown and harvested as described before (9) and the lipids were isolated by the same procedure (complete extraction of lipids from *Saccharomyces cerevisiae* required prior rupture of the cell walls in the Ribi-Sorvall cell fractionator).

Materials

Cyclohexylamine, DMEA, MMEA, and *t*-butanol were purchased from Distillation Products Industries (Rochester, N.Y.). EA was purchased from the Fisher Chemical Corp. (St. Louis, Mo.). GLC analysis indicated that these reagents were sufficiently free from contaminants to render further purification unnecessary. GLC materials were purchased from the Applied Science Laboratories Inc. (State College, Pa.) and the F & M Scientific Division of Hewlett-Packard Co. (Avondale, Pa.).

Liberation of Bases by Acid Hydrolysis

Intact or deacylated phospholipids in screw-cap centrifuge tubes containing up to 15 μmoles of phosphorus were taken to dryness in a stream of nitrogen. 2 ml of aqueous 6 M HCl was added and the Teflon-lined caps of the tubes were tightly closed. Hydrolysis proceeded at 100°C for 3 hr. The hydrolysate was cooled and the cyclohexylamine internal standard added. Usually 1–10 μl of a 0.2 M solution gave the desired ratio of 1 μmole of cyclohexylamine per 10 μmoles of EA. After the solution had been saturated with solid anhydrous sodium carbonate, 1 ml of *t*-butanol was added and the mixture was vigorously agitated with a vortex mixer for several minutes. The emulsion was then centrifuged in a clinical centrifuge and 2–5 μl of the butanol phase was injected into the gas chromatograph.

Numerous water-immiscible solvents were tried that would both extract and neutralize the amine hydrochlo-

Abbreviations: GLC, gas–liquid chromatography; EA, ethanolamine; DMEA, dimethyl ethanolamine; MMEA, monomethyl ethanolamine.

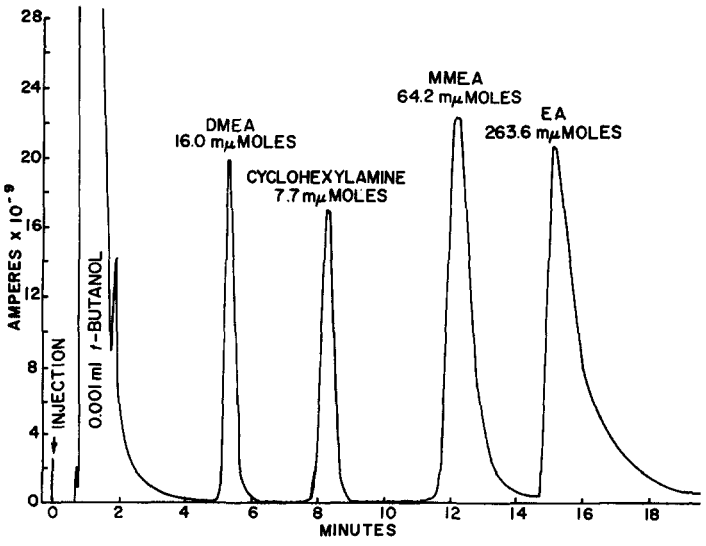

FIG. 1. Chromatogram of a standard mixture of amines treated with acid, neutralized, extracted into *t*-butanol, and subjected to GLC by the standard procedure.

rides. Organic amines proved either too impure even after careful distillation or to have retention times that overlapped those of the bases. Attempts were also made to evaporate the hydrolysate to dryness and redissolve the amine hydrochlorides in alcoholic base. Methanol, ethanol, isopropanol, and *n*-propanol containing sodium hydroxide would neutralize and dissolve the amine hydrochlorides but gave tailing in GLC that interfered with the quantitative estimation of DMEA. However, this procedure was found to be valuable in the quantitative estimation of EA in deacylated phospholipid mixtures. In this case the cyclohexylamine internal standard was added to the HCl hydrolysate which was then evaporated to dryness in a stream of nitrogen. Just enough 0.33 N KOH in methanol containing a trace of bromothymol blue was added to give the solution a blue color. The solution of the free amines was then directly injected into the gas chromatograph. This procedure results in an approximately 5-fold increase in sensitivity over the *t*-butanol extraction method for EA.

Analysis by GLC

A dual column gas chromatograph (F & M Scientific Division, Hewlett-Packard Corp., Model 402) with hydrogen flame detectors was used in this study. U-shaped glass columns of 3 mm I.D. and 1.8 m long were used. The columns were packed with silanized, acid-washed Chromosorb P, 80–100 mesh, coated with 25% Dowfax 9N9 (Applied Science[1]) and 2.5% sodium hydroxide as described by O'Donnell and Mann (10). This column has an efficiency for cyclohexylamine of 1330 theoretical plates. The chromatography was performed isothermally at 88°C with the flash heater at 100°C and the detectors at 200°C. The carrier gas was helium at a pressure of 60 psi and a flow rate of 162 ml/min. Hydrogen at a pressure of 18 psi and a flow rate of 70 ml/min and air at a pressure of 30 psi and a flow rate of 485 ml/min were set so as to optimize the detector response. Under the chosen conditions 1 mμmole of cyclohexylamine gave a peak response of 7.15×10^{-10} amp in the hydrogen flame.

This column should be kept capped when not in use and activated with helium flowing at 150°C for at least 6 hr before use.

Modifications of the solid support in which less active Diatoport S or glass beads were used or reduction of the content of Dowfax 9N9 were not effective in separating the ethanolamines although tailing of the solvent and EA were reduced. Other stationary phases gave less satisfactory separations. Those tested included Carbowax 20M, Carbowax 2000, Epon 1001, SE-30, SE-52, and ethylene glycol succinate. The presence of the sodium hydroxide in the liquid phase was essential. Treating the glass wool used to plug the columns with sodium hydroxide gave no improved separations.

RESULTS

The chromatogram reproduced in Fig. 1 shows the response of the GLC system to a mixture of MMEA, DMEA, EA, and cyclohexylamine. The retention time of each component was established by injecting it alone. The retention times are reproducible. Cyclohexylamine was chosen as an internal standard as it gives a symmetrical peak, has a retention time between those of

[1] Dowfax 9N9 is a nonionic surfactant made from nonyl phenol condensed with 9–10 moles of ethylene oxide.

DMEA and MMEA, and can be used directly as supplied commercially.

Extraction of the Hydrolysate

To be an effective assay of phospholipids, the method must include a means of extracting the ethanolamines as free bases from a hydrolysis mixture. Portions of various known mixtures of the four amines were acidified with HCl, saturated with anhydrous sodium carbonate, and extracted with *t*-butanol as described in Materials and Methods. Portions of these known mixtures of the free bases were also added directly to the *t*-butanol. A comparison of the two butanol solutions by GLC showed that the extraction is quantitative. If the aqueous solution is not saturated with sodium carbonate the EA is not completely extracted.

Detector Response

The linearity of the detector response to the four amines is illustrated by Fig. 2. The response of the detector to all the amines is linear between concentrations of 0.9 mμmole and 150 mμmoles to within ±0.5%. Because of the asymmetry of the peaks, especially for EA, they were measured by cutting them out and weighing the paper. Weights of equal areas of chart paper were equal within 1%. Determination of the areas with a disc integrator proved equally accurate.

The relative response of the hydrogen flame detector to each of the components is 100:48.2:30.1:11.2 for cyclohexylamine : DMEA:MMEA : EA. A plot of the relative response per mole versus the number of carbon atoms is shown in Fig. 3. There is a linear increase in the relative response for EA, MMEA, and DMEA that is displaced from the origin about 1.35 carbon units. A line of the same slope intersecting the relative response of cyclohexylamine is displaced 0.7 carbon units from the origin. From the data of Perkins, Rouayheb, Lively, and Hamilton (11) the response would be expected to be displaced about 1.2 carbon units from the origin (0.7 for the amine and 0.5 for the alcohol).

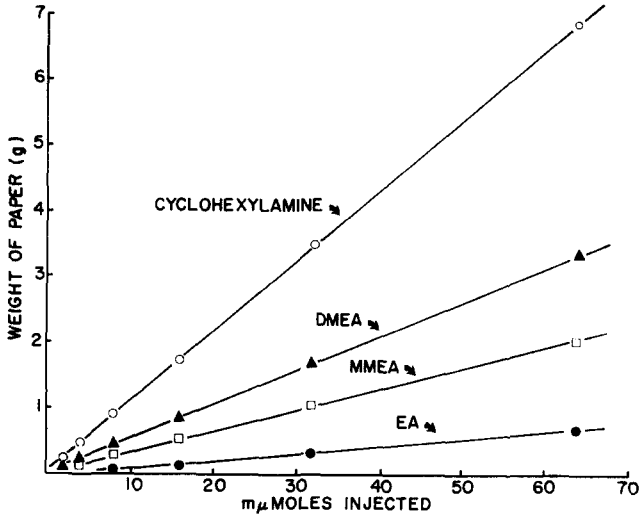

FIG. 2. Response of the hydrogen flame detector to increasing molar quantities of EA, MMEA, DMEA, and cyclohexylamine during GLC.

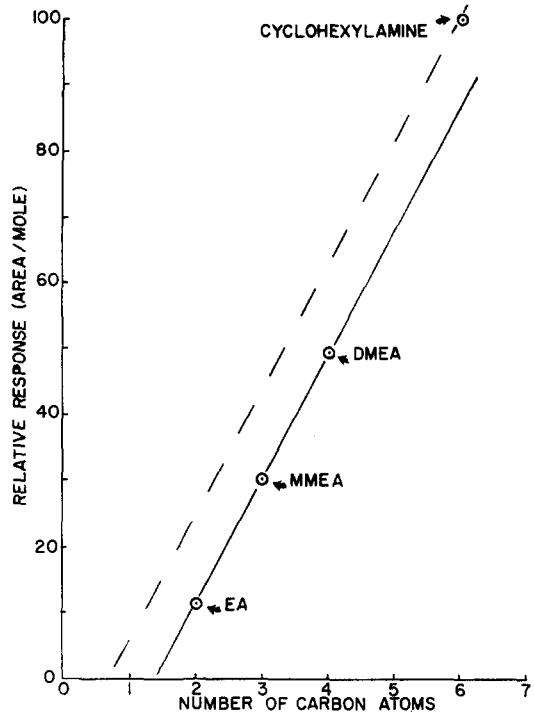

FIG. 3. Relative molar response of the hydrogen flame detector per carbon atom of EA, MMEA, DMEA, and cyclohexylamine.

Reproducibility

The use of an internal standard to correct for injection errors results in a reproducibility of about 1% for the entire assay procedure. With this rapid, quantitative method the EA, MMEA, and DMEA content of several tissues was examined. The results are given in Table 1.

TABLE 1 EA, MMEA, AND DMEA CONTENT OF RAT, BACTERIAL, AND YEAST LIPIDS

	EA	MMEA	DMEA
		% of total phospholipid	
Rat brain	18.1	<0.02	<0.02
Rat heart	24.7	0.16	<0.02
Rat liver	21.3	<0.02	<0.02
Rat muscle	14.1	0.39	<0.02
Yeast	11.2	1.64	3.24
Hemophilus parainfluenzae	68.4	0.35	<0.02

About 15 μmoles of lipid phosphorus from each source was prepared for GLC. 5–65 mμmoles of EA was in each 5 μl sample subjected to chromatography.

DISCUSSION

Previous methods (2–5) for the resolution and estimation of the ethanolamine-containing lipids have involved the following procedures: column chromatography on various adsorbents, hydrolysis of the eluates, separation of the amines by ion-exchange chromatography or paper chromatography, elution, treatment with methyl iodide in base, and formation of the reineckate salt. The GLC method described here is applicable to unfractionated lipid extracts. If combined with fraction collection it could easily be used to determine the specific radioactivities of the parent lipids. The method is at least as sensitive as any previously reported. As little as 0.20 μmole of EA can easily be detected in the 1 ml of *t*-butanol. The sensitivity could be improved both by preliminary fractionation of the lipids and by reducing the volume of the hydrolysis mixture and the *t*-butanol extract.

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