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High-performance liquid chromatography method with light-scattering detection for measurements of lipid class composition: analysis of brains from alcoholics

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Abstract

A high-performance liquid chromatographic method with evaporative light-scattering detection was developed for the analysis of intact lipid classes in nervous tissue. The method had the ability to resolve plasmalogen-phosphatidylethanolamine and diacyl-phosphatidylethanolamine along with other major phospholipid classes in a single run. This technique was employed for the investigation of the effects of chronic alcohol consumption on the membrane lipid class composition of human brains (alcoholics, $n=13$; controls, $n=11$). Measurements were performed on cholesterol, cerebrosides, sulfatides, phospholipids and sphingolipids in total lipid extracts of white matter, gray matter and cerebellar regions of human brains. No significant differences in the lipid class composition between the groups were observed.

Keywords: Lipids; Cholesterol; Sulfatides; Phospholipids; Sphingolipids

1. Introduction

Acute ethanol administration affects several neurotransmitter systems [1,2] and prolonged alcohol intake may cause structural changes in the brain, such as shrinkage, as measured by an increase in the pericerebral space [3–6]. Chronic alcohol consumption alters lipid metabolism [7] and may lead to a variety of compositional alterations in the nervous system [8–13]. Nervous tissue membrane lipid composition (for review, see Refs. [14,15]) appears to be more resistant to alcohol exposure [16] than those of other organs but studies have been performed which shows a decrease in 22:6n3 of mouse myelin phospholipids after ethanol exposure [17]. In order to investigate the impact of chronic alcoholism on the lipid class composition of human brain, the present investigation was conducted.

HPLC methods for the separation of intact lipids from brain tissue have been published [18,19] using chlorinated solvents and acid or base containing mobile phases but fail to resolve plasmalogen-phosphatidylethanolamine (plasmalogen-PE) from diacyl-phosphatidylethanolamine (diacyl-PE). Alkenyl and alkyl group compositions can be determined after rather time-consuming hydrolysis and derivatization reactions [20] or, alternatively, with LC–MS [21]. In this study, a method was needed that would separate

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both forms of PE without derivatization steps and in a single chromatographic run. To this end, high-performance liquid chromatography (HPLC) was used in conjunction with an evaporative light-scattering detector [18,19,22–25], employing a solvent system suitable for subsequent preparative chromatography of individual lipids classes for more detailed analysis. This technique was employed for the investigation of the effects of chronic alcohol consumption on the membrane lipid classes of human brains. Measurements of cholesterol, cerebrosides, sulfatides, phospholipids and sphingolipids in total lipid extracts were performed on white matter, gray matter and cerebellum.

2. Experimental

2.1. Case selection

The material used in this study was obtained from forensic autopsies at the New South Wales Institute of Forensic Medicine and the Royal Prince Alfred Hospital. Classification of cases into control and alcohol groups were based on compilation of clinical and pathological data. The data included clinical notes from teaching hospitals as a result of previous admissions, telephone interviews with general practitioners and relatives of the subjects, reports concerning circumstances of death, and a complete necroscopy with microscopic examination of tissues including the liver and the brain. The alcoholics consumed greater than 80 g of ethanol/day for a mean duration of 25 years (range 7–45 years). Classification of a case as 'alcoholic' was based on DSM IV diagnostic criteria for substance dependence [26]. Written consent for autopsy was obtained for hospital cases and the study approved by the Human Ethics Committee of the University of Sydney and the Royal Prince Alfred Hospital under the New South Wales Transplantation and Anatomy Act, as well as the Office of Human Subjects Research (NIH, Bethesda, MD, USA). Patients with a history of significant neurological diseases other than those associated with alcoholism were excluded. Any patient with macroscopic evidence of head injury was excluded from the study, as were control subjects in whom there was a history of alcohol intake greater than 20 g of alcohol per day. Liver steatosis was observed in seven, and cirrhosis in three of the alcoholic subjects. The most common hepatic pathology in controls was hepatic congestion. Subjects were sex and age-matched between groups. The total number of subjects was 24, of which 13 were diagnosed with chronic alcoholism and 11 were regarded as controls. The control and alcoholic cases were coded prior to analysis and the code broken only after completion of the analytical determinations.

2.2. Tissue preparation

As soon as possible after autopsy (usually within one hour of removal from the calvarium), each brain was patted dry and the weight recorded. The brainstem and cerebellum were separated from the cerebrum at the level of the superior colliculus. The hemispheres were divided sagitally by cutting between the mammillary bodies, and through the commissures and corpus callosum. The left hemisphere was left intact and fixed by suspension in 15% formalin for routine neuropathological examination. The right hemisphere was divided by coronal sections at the level of the anterior border of the temporal pole and the posterior border of the thalamus. Meninges were removed with forceps from the cortical areas being sampled. Areas sampled were, (1) a block from the lateral cerebellum (CB), (2) a block of the parieto-occipital white matter (WM), and (3) cortical gray matter (GM) obtained by scraping a scalpel blade over the cortex from several adjacent parieto-occipital blocks. All tissues were placed into resealable labelled plastic bags and snap frozen by partial immersion of the plastic bag in 100% ethanol at −70°C. Tissue was stored at −70°C until lipid extraction.

2.3. Extraction

Samples of nervous tissues were extracted in a randomized order, utilizing chloroform, methanol and water according to Bligh and Dyer [27]. The samples were homogenized using a Polytron homogenizer in test tubes with PTFE-lined screw caps; 200 mg/l of butylated hydroxytoluene (BHT) was added. Immediately before extraction, argon was

bubbled through the solvents to remove oxygen. After homogenization and extraction, samples were filtered through a 0.45-μm filter (MILLEX-HA, Millipore, Bedford, MA, USA), sealed under nitrogen and stored at −80°C until analysis.

2.4. Standards and solvents

The lipid class standard Spectralipid SN [28], was obtained from Spectral Service (Cologne, Germany). Sulfatide cerebroside was purchased from Sigma (St. Louis, MO, USA). All other lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

2.5. Lipid class analysis by HPLC

The solvent system developed by Arnoldsson and Olsson [29] was used for the HPLC separation of brain lipid classes. However, in this investigation a different analytical column was used. Also, the flow-rate, temperature of analysis and gradient profile were modified to meet the special needs of this investigation, i.e., separation of plasmalogen-PE as well as phosphatidic acid (PA) and phosphatidylserine (PS) which were not of interest in the original method paper [29]. The chromatographic system consisted of a Hewlett-Packard HP 1090 liquid chromatograph with a column oven set at 65°C. The analytical column was a Nucleosil 100 - 7 OH DIOL (250×4.0 mm I.D., 7 μm) purchased from Macherey-Nagel (Düren, Germany). The effluent from the column was connected to a Sedex 55 light-scattering detector (S.E.D.E.R.E., Alfortville, France). The detector inlet pressure was 2.3 bar (nitrogen) and the drift tube temperature was 65°C. Gradient control as well as integration was performed on an HP 7957B workstation. Solvent A was hexane–2-propanol–*n*-butanol–tetrahydrofuran–isooctane–water, (64.5:17.5:7:5:5:1, v/v) and solvent B was 2-propanol–*n*-butanol–tetrahydrofuran–isooctane–water, (73:7:5:5:10, v/v); 180 mg NH_4Ac/l was added to both solvents. The binary solvent gradient was linear over 50 min, from 0% to a final composition of 100% B.

In order to exclude any time-dependent variations in the data, the samples were run in a randomized order. Calibration was done with external standards at six dilutions, utilizing the Spectralipid SN standard for PE, phosphatidylinositol (PI), PA and phosphatidylcholine (PC) (Fig. 1). Other peaks were calibrated against individual standards. The light-scattering detector exhibits a narrower linear range than does, for example a UV detector, but herein linear calibration curves could be used for the major phospholipid classes. Quantitation of PC, for example, was achieved by a linear calibration curve (r^2= 0.997) in the range of 15–98 μg. In order to maintain peaks within the calibrated range, the

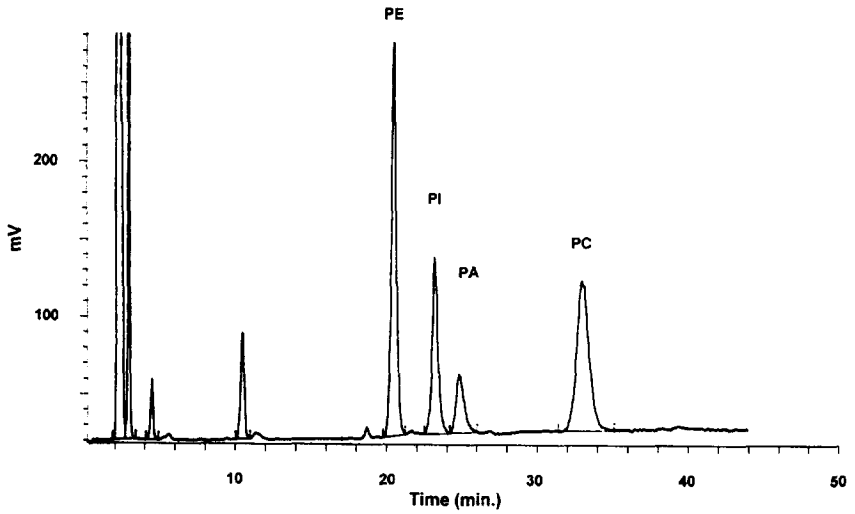

Fig. 1. HPLC profile of the Spectralipid SN standard mixture monitored by light-scattering detection. For conditions see Section 2.

amount of brain tissue total lipid extract injected was 275 μg.

3. Results and discussion

The HPLC method developed in this investigation is suitable for lipid compositional studies of biological material from many sources. The mobile phases contain no chlorinated or aromatic solvents and no acid or bases. This approach helps to prevent the degradation of plasmalogen linkages. Baseline resolution was achieved between all lipid classes with partial resolution between alkenylacyl-PE and diacyl-PE (Fig. 2). The resolution was adequate for a higher column sample load (approx. 500 μg), desirable for efficient preparative chromatography of individual lipid classes for further analysis. The identities of alkenylacyl-PE and diacyl-PE peaks were confirmed, after HPLC fractionation followed by methylation with BF_3 [30] and fatty acid analysis by gas chromatography. Dimethylacetal derivatives (from alkenyl–glycerols) were absent in the diacyl-PE fraction and present in the alkenylacyl-PE fraction.

The DIOL phase column was notably resistant to retention time shifts. No significant loss in resolution between the phospholipid and cerebroside peaks was observed after more than 1000 injections. The mean relative standard deviation, as shown in Table 1, was 5.4%. Usage of the evaporative light-scattering detector ensures that the mass of each lipid class, regardless of degree of unsaturation, is detected with similar sensitivity, as opposed to methods employing UV detection [25,31]. As the chromatographic conditions of the present method were stable and reproducible, high accuracy and precision of the determinations were generally attained by an individual six level calibration curve for each lipid class.

The average wet weight of the WM, GM and CB tissue pieces extracted and their mean total lipid content was 1.48 g (19.0%), 1.58 g (5.4%) and 2.53 g (5.6%), respectively. The analysis of the total lipid extracts did not show any significant changes in lipid class composition when alcoholic samples were compared to controls in any of the three brain regions under study (Table 2). No gender differences were detected and therefore male and female subject data were pooled for each group.

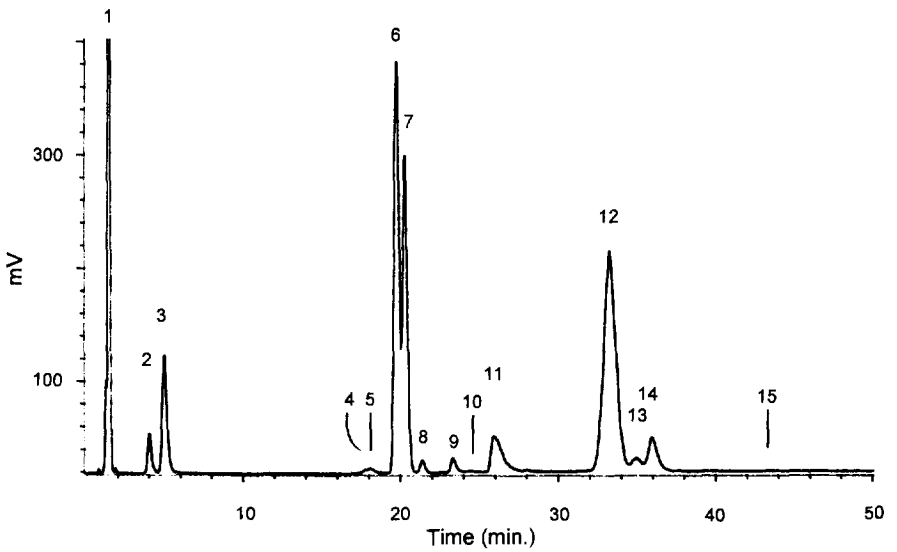

Fig. 2. HPLC chromatogram of human brain cerebellum total lipid extract monitored by light-scattering detection. For conditions see Section 2. Peaks: 1=cholesterol, 2=cerebroside, 3=hydroxylated cerebroside, 4=sulfatide, 5=hydroxylated sulfatide, 6=plasmalogen-PE, 7=diacyl-PE, 8=unknown, 9=PI, 10=PA, 11=PS, 12=PC, 13=sphingomyelin-1, 14=sphingomyelin-2, 15=lyso-PC.

Table 1
Reproducibility of HPLC method using a phospholipid standard[a]

Lipid	Stated composition (weight%)	Measured composition[b] (area%)	S.D.	R.S.D.[c] (%)
PE	12.1	16.5	0.77	4.7
PI	9.6	10.0	0.54	5.4
PA	5.4	4.8	0.28	5.8
PC	14.4	14.2	0.78	5.5

[a]Representative phospholipids included in the Spectralipid SN standard mixture.
[b]Average of six chromatographic runs of the same sample preparation run on six consecutive days ($n_{tot}=6$).
[c]Relative standard deviation. Overall method reproducibility was 5.4%.

Chronic alcoholism affects different tissues to varying degrees and liver and plasma are among those most easily disturbed. In comparison, brain lipids appear more resistant to stress by alcohol [13]. Ethanol treatment had reportedly no effect on total phospholipid levels in mouse brain [16]. The adult brain also show a marked resistance to changes in lipid composition during nutritional challenge [32,33]. Accordingly, the results of the present investigation shows that alcohol exposure has little effect on human brain tissue phospholipid distribution in the three brain regions investigated.

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Table 2
Human brain lipid class composition[a]

Class[b]	Cerebellum		Gray matter		White matter	
	Control ($n=11$) (wt.%)	Alcoholic ($n=13$) (wt.%)	Control ($n=10$) (wt.%)	Alcoholic ($n=13$) (wt.%)	Control ($n=11$) (wt.%)	Alcoholic ($n=13$) (wt.%)
Cholesterol	21.7±2.94	21.1±3.85	19.6±3.17	20.5±5.22	26.9±5.79	27.5±2.99
Cerebroside	2.9±1.11	2.8±0.96	2.9±1.51	2.8±1.16	8.3±1.77	9.2±1.71
Cerebroside-OH	4.3±0.98	4.3±1.36	4.3±2.37	4.4±1.48	10.1±2.40	11.5±2.33
Sulfatides[c]	0.7±0.40	0.6±0.30	0.7±0.34	0.7±0.40	5.1±1.23	4.6±1.10
Plasmalogen-PE	16.0±1.71	15.8±1.93	13.3±0.55	13.8±0.70	19.6±2.13	18.6±1.34
Diacyl-PE	13.0±1.89	13.3±1.61	17.4±2.83	16.1±1.76	–[d]	–[d]
PI	3.4±1.14	3.4±1.07	3.9±1.36	3.6±1.24	4.0±1.25	4.1±0.89
PA	0.2±0.03	0.3±0.12	0.5±0.35	0.3±0.13	0.4±0.20	0.3±0.05
PS	7.0±1.29	6.6±1.16	7.2±1.57	7.5±1.19	9.0±1.20	8.8±0.71
PC	25.7±2.24	26.3±3.01	25.1±3.31	25.4±2.40	11.8±1.43	10.9±0.80
SPH-1	1.0±0.33	1.0±0.25	1.0±0.28	0.7±0.10	3.1±0.72	2.9±0.30
SPH-2	2.4±0.79	2.5±0.77	2.2±0.60	2.4±0.46	1.3±0.37	1.1±0.23
LPC	0.5±0.73	0.2±0.08	0.3±0.25	0.2±0.13	–	–
Unidentified	2.0±1.11	2.4±2.17	2.5±0.97	2.3±1.69	0.8±0.99	0.7±0.46

[a]Values expressed as the mean wt. %±S.D.
[b]Cerebroside-OH=cerebrosides containing hydroxylated fatty acids, PE=phosphatidylethanolamine, PI=phosphatidylinositol, PA=phosphatidic acid, PS=phosphatidylserine, PC=phosphatidylcholine, SPH-1 and SPH-2=two forms of spingomyelin differing in N-acylated chain length, LPC=lyso-phosphatidylcholine.
[c]Sulfatide and hydroxy sulfatide grouped together.
[d]Contains approximately 5% diacyl form which could not be well resolved in white matter due to its limited content.

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