An improved method for the identification and quantitation of biological lipids by HPLC using laser light-scattering detection

B.S. Lutzke and J.M. Braughler

The Upjohn Company, CNS Research and Development, Kalamazoo, MI 49001

Summary We have developed a simplified and improved high performance liquid chromatography (HPLC) method for the detection and quantitation of tissue lipid using a new laser light-scattering detector (Varex model ELSD II). This detector has a limit of sensitivity of 50 ng for neutral lipid and 200 ng for most phospholipids with excellent reproducibility. By coupling the ELSD II with a ternary gradient normal phase HPLC system, we were able to separate and quantify the major lipid constituents of extracted tissue. This system was used to profile and quantitate the major lipids from rat brain, liver, and heart with greater sensitivity than other available techniques, with the exception of high performance thin-layer chromatography (HPTLC). However, the convenience of HPLC allows for a significant improvement in analysis time with only a threefold reduction in sensitivity when compared to HPTLC.—Lutzke, B. S., and J. M. Braughler. An improved method for the identification and quantitation of biological lipids by HPLC using laser light-scattering detection. *J. Lipid Res.* 1990. **31:** 2127–2130.

Supplementary key words lipid analysis

Prior to 1985, the detection and quantification of the lipid content of animal tissue by HPLC was a difficult and imprecise procedure. Although baseline separation of lipids was possible, UV quantitation was not practical since absorbance recordings arose primarily from double bonds in the fatty acid moieties (1), and molar extinction coefficients vary with fatty acid composition. The fatty acid composition of esterified lipids may change with different metabolic states and tissue types, forcing experimental conditions to be strictly controlled to obtain reproducible results. In addition, previous methods of detection in many cases precluded the use of multiple gradient systems for optimal component separation. The advent of a light-scattering detector used in conjunction with a multisolvent HPLC gradient overcame some of the deficiencies of the previous methods. Greater sensitivity was achieved, lipids could be detected and quantified in a single HPLC run, and analysis time was significantly reduced (2).

The principle of operation of a light-scattering detector is based on conversion of the effluent from the column into a fine mist by passing it through a nebulizer into a stream of nitrogen gas. The nebulized lipid droplets fall through a heated "drift" tube which evaporates the volatile portion of the effluent. The remaining lipid droplets scatter a beam of light when they travel through its path. The response of this detector is then a function of the mass of the lipid in the droplets passing through the detector, in contrast to most HPLC detection systems whose response is a function of the concentration of the solute. The ELSD II, whose use in lipid analysis is described in this report, is the latest improvement on this detector. We find that, for lipid analysis, the limit of detection is much greater than for previous systems, reproducibility is better from run to run, and there is less difficulty with aqueous solvent. The improved ELSD II detector, however, offered little advantage over the older ACS light-scattering detector when coupled with previously described mobile gradient systems (Christie, 2, 3). Accordingly, we have devised a new ternary gradient system that yields enhanced lipid separation and mass sensitivity, especially for the polar phospholipids. In addition, the column life is extended due to lower operating pressures experienced during the run, particularly in the central part of the gradient where the aqueous solvent tends to increase pressure.

METHODS

Materials

Phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), sphingomeylin (SM), lysophosphatidylethanolamine (LPE), lysophosphatidylcholine (LPC), sulfatide (SUL), and cardiolipin (CL) were purchased from Matreya, Inc. (Pleasant Gap, PA). Nonhydroxy and hydroxy ceribrosides (CER-1 and CER-2, respectively), triolein (TG), cholesteryl oleate (CE), oleic acid (FA), and cholesterol (CHOL) were supplied by Serdary Research Laboratories (London, Ontario, Canada). N-oleylethanolamine (NOE) was synthesized (4) and graciously supplied by D.E. Epps of the Upjohn Company. Isopropanol, methanol, chloroform, and iso-octane were from Burdick and Jackson, and tetrahydrofuran was from Sigma (St. Louis, MO). All other materials were the highest grade commercially available.

Tissue preparation

Sprague-Dawley rats (140–150 g) were killed by decapitation. Tissues were removed and homogenized immediately in ice-cold Krebs (5) buffer (1:5, w/v) with a Wheaton dounce tissue grinder. Lipid extracts were prepared using a modified Folch extraction (6). Briefly, 150 μl of homogenate was diluted to a final volume of 1.5 ml with Krebs buffer, and 2 ml of methanol, containing 10 μg/ml NOE as an internal standard, was added. Samples were vortexed for 10 sec, 4 ml of chloroform was added, and the samples were vortexed again for 10 sec, followed by centrifugation for 5 min at 1500 g (4 °C) to facilitate phase separation. The lower organic phase was transferred to a clean glass cul-

Abbreviations: HPLC, high performance liquid chromatography.

TABLE 1. Gradient and flow program used by the Perkin-Elmer Series 4 pump for the lipid separation method

Sector	min	ml/min	Flow Curve[e]	IO/TH[b] % A	IPA/C[c] % B	IPA/W[d] % C	Gradient Curve[e]
Equil[a]	5.0	2.0		100	0	0	
1	1.0	2.0		100	0	0	
2	4.0	2.0		80	20	0	1.0
3	4.9	1.6	1.0	44	52	4	1.0
4	0.1	1.5	0.0	42.5	52	5.5	0.5
5	10.0	1.5		32.5	52	15.5	5.0
6	6.7	1.5		29	52	19	1.0
7	0.1	1.5		30	70	0	1.0
8	6.5	2.0	5.0	100	0	0	1.0
9	0.0						

[a]Equil, equilibration time.
[b]IO/TH, iso-octane-tetrahydrofuran 99:1 (v/v).
[c]IPA/C, isopropanol-chloroform 4:1 (v/v).
[d]IPA/W, isopropanol-water 1:1 (v/v) (note: the water contained 500 μM serine adjusted to pH 7.5 with ethylamine to aid in detection of the more acidic phospholipids).
[e]These values correspond to the shape of the gradient and flow rate curves used by the Perkin-Elmer Series 4 pump system.

ture tube and the upper phase was re-extracted as above using an equivalent volume of theoretical lower phase of chloroform-methanol-water 86:14:1 (v/v/v). The lower phase extracts were combined, dried under a stream of argon, and redissolved in 200 μl iso-octane-chloroform 1:1 (v/v) containing 0.05% butylated hydroxytoluene (BHT).

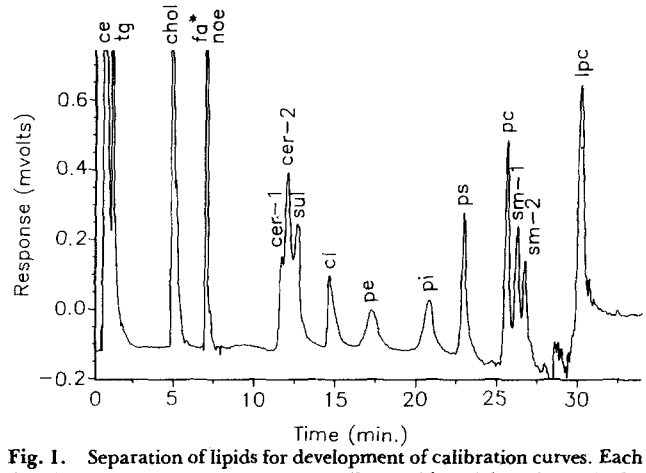

Fig. 1. Separation of lipids for development of calibration curves. Each lipid is present at 1 μg amounts except fatty acid (FA) (not shown*); the internal standard was n-oleylethaolmine (NOE). Elution conditions are described in the methods section. Other abbreviations: CE, cholesteryl ester; TG, triglyceride; CHOL, cholesterol; CER-1, nonhydroxy cerebroside; CER-2, hydroxy cerebroside; SUL, sulfatide; CL, cardiolipin; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine: SM-1, nonhydroxy sphingomeylin; SM-2, hydroxy sphingomeylin; LPC, lysophosphatidylcholine.

Lipid standards and lipid quantitation

Lipid standard curves were generated from a total lipid standard dissolved in iso-octane-chloroform 1:1 (v/v) containing 0.05% BHT. Dilutions were prepared from a stock

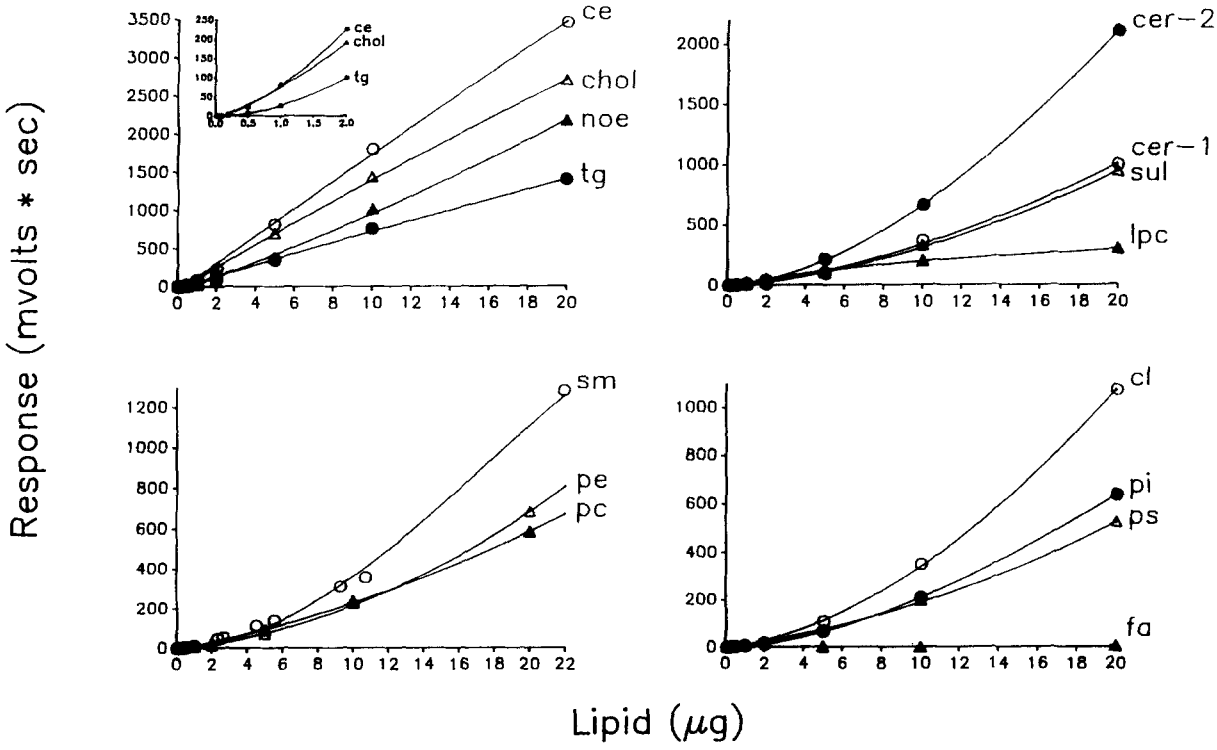

Fig. 2. Calibration curves of weight of each lipid injected versus detector response (mvolts*sec). Each point represents a single injection. Lipids are grouped according to sensitivity to detection with the neutral lipids yielding the greatest sensitivity and the phospholipids the least. The inset graph is an expansion of the CE, CHOL, and TG calibration curves from 50 ng to 2 μg.

total lipid standard containing 5 μg/ml each of: CE, TG, CHOL, NOE, CER-1, CER-2, SUL, CL, PE, PI, PS, PC, LPC, and SM. The SM eluted as two peaks (SM-1 and SM-2) in our system, presumably due to esterified nonhydroxy and hydroxy fatty acids. We assumed that both SM species had approximately the same detector response and determined their response curve accordingly. Ten-microliter samples, which contained 5.0, 2.0, 1.0, 0.5, 0.2, 0.1, 0.05, 0.02, 0.01, or 0.005 μg/μl of each lipid standard, were injected. Peak responses were measured and quantified using Maxima 820 software developed by Dynamic Solutions. Baseline values were subtracted from all standard and sample chromatograms. NOE, which is not endogenous at detectable levels in normal tissue extracts, was used as an internal standard.

HPLC and detection system

Samples were chromatographed on a S3W Spherisorb, 10 cm × 4.6 mm, 3 μm silica, Phase Sep column. The guard column was a Waters RCSS Guard-PAK silica column. Lipid detection was achieved using a Varex ELSD II laser light-scattering detector, with the nitrogen gas flow set at 48–50 mm and a gas pressure of 22 psi. The drift tube was heated to 115 °C and the exhaust gas temperature was 73 °C. The chromatography system was a Perkin-Elmer Series 4 liquid chromatograph equipped with a Perkin-Elmer ISS-100 multi-sample auto-injector with a Rheodyne valve and 10-μl sample loop. The mobile phase was developed using a gradient modified from that reported by Christie (3), and our modified gradient included a flow rate gradient that served to maintain low column pressure and enhance detector response to the phospholipids. The specific ternary gradient and flow program used was as described in **Table 1**.

RESULTS AND CONCLUSIONS

Using our method as described, we could easily detect 1 μg or less of any of the neutral or polar lipids used as standards. A typical chromatogram of 1 μg of each of the standard lipids (**Fig. 1**) indicates that detector response varied for the different lipids. Neutral lipids exhibited the greatest response on a μg/mvolt basis with a limiting sensitivity of 50 ng. As expected, detector response to phospholipids was less than for neutral lipids inasmuch as phospholipid peaks were broader and eluted in the more aqueous portion of the gradient. Nevertheless, even PS, which had low detector response, was detected in sub-microgram quantities. NOE and FA co-eluted in our system. Since FA was not detectable in our tissue extracts in appreciable amounts, we opted to use NOE as our internal standard. If the presence of significant quantities of FA is a concern, a different internal standard can be chosen or a radioactive marker added to monitor recovery.

Standard curves for use in the quantitation of the extracted lipids were individually generated for each lipid in

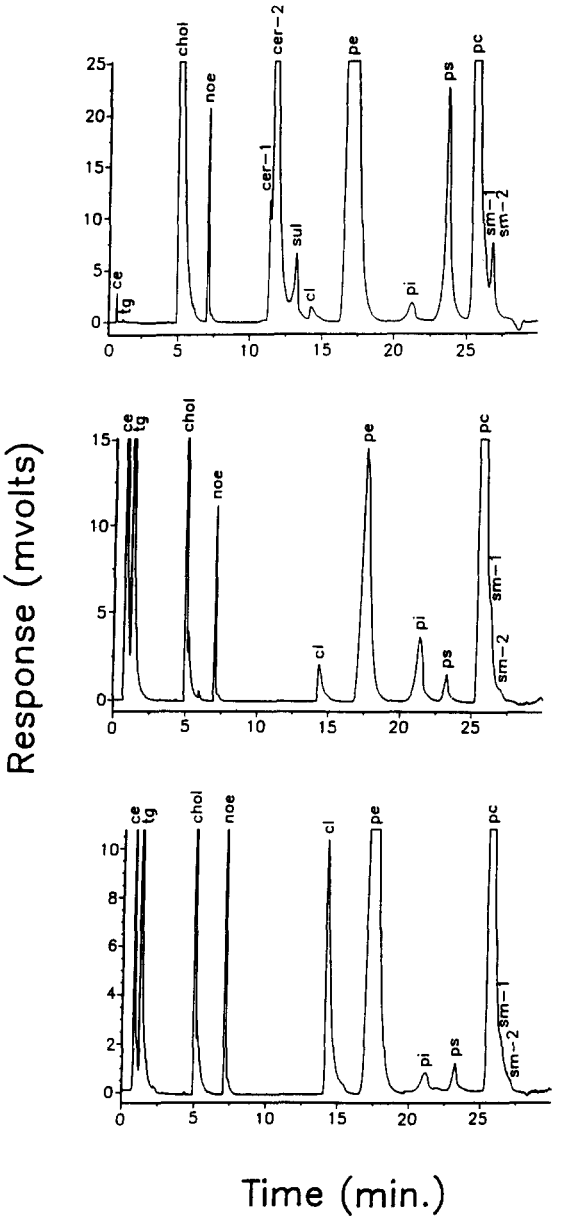

Fig. 3. Separation of the lipids of three rat tissue extracts. The individual lipids of these samples are quantitated in Table 2. The top chromatogram is 200 μg of lipid extract from the brain, the middle represents 100 μg of liver extract, and the bottom is 82 μg of heart lipid.

of the standard points for each lipid with a log-weighted cubic fit routine that is part of the Maxima 820 software package. Although we cannot directly compare the limits of sensitivity of our system with the method of Christie (2), some qualitative comparisons may be made. Our standard curves for the majority of the lipids are reliable down to 200 ng while it appears that standard curves published previously for the ACS detector are uncalibrated below 10 μg (2). We also demonstrate (**Fig. 3**) that for the heart lipid extract,

TABLE 2. A comparison of the quantitation of rat tissue lipids to values obtained by Christie (2) and Wells and Dittmer (7)

Lipid	Heart		Liver		Brain	
	Present Method	Christie	Present Method	Christie	Present Method	Wells & Dittmer
	% of total lipid		*% of total lipid*		*μmol/g tissue*	
CE	2.31 ± 0.008	0.22 ± 0.042	3.80 ± 0.018	1.47 ± 0.102	0.128 ± 0.007	
TG	6.49 ± 0.004	3.77 ± 0.231	8.89 ± 0.019	6.66 ± 0.151	0.113 ± 0.008	
CHOL	4.00 ± 0.016	4.06 ± 0.159	3.78 ± 0.017	5.40 ± 0.130	38.824 ± 0.357	39.5
CER (total)					12.013 ± 0.103	18.6
CER-1					3.386 ± 0.086	
CER-2					8.645 ± 0.057	
SUL					3.314 ± 0.040	3.2
CL	10.20 ± 0.001	12.30 ± 0.403	3.58 ± 0.026	4.54 ± 0.045	0.952 ± 0.012	0.7
PE	26.80 ± 0.015	33.44 ± 0.209	11.12 ± 0.008	19.91 ± 0.080	25.111 ± 0.029	24.4
PI	3.17 ± 0.003	3.69 ± 0.005	7.40 ± 0.008	4.43 ± 0.077	2.391 ± 0.015	2.3
PS	2.81 ± 0.007		2.90 ± 0.006		10.831 ± 0.049	8.3
PC	39.20 ± 0.010	38.57 ± 0.203	46.64 ± 0.010	55.18 ± 0.126	28.707 ± 0.040	25.2
SM (total)	4.64 ± 0.079	1.76 ± 0.502	5.76 ± 0.059	2.09 ± 0.060	5.572 ± 0.140	3.6
SM-1	2.92 ± 0.059		4.40 ± 0.116		2.523 ± 0.137	
SM-2	1.72 ± 0.052		1.36 ± 0.057		3.049 ± 0.029	

Values for the present method are the mean ± standard error of three injections of the same sample. Values are expressed as a percent of the total lipid weight for heart and liver, and μmol lipid/g tissue for the brain.

80 μg of total lipid is more than sufficient to obtain accurate quantitation of the major lipids. This sensitivity is 4- to 5-fold greater in terms of mass than previously shown (using a similar method of detection) for heart lipids (3). Although we were able to determine the retention time for standard LPE (22.2 min), we did not quantify it since it was present at levels below our detection limits in the small samples of tissues we examined.

This methodology as described has proven to be highly reproducible and yields results that are comparable with previously reported lipid estimates. **Table 2** shows the quantitation of various lipid extracts compared with those reported by Christie (2) and Wells and Dittmer (7). We report the mean and standard error of three injections of each tissue extract. Our data show that this method yields results that agree closely with values reported in the literature by others. We are not able to separate the diacyl and plasmalogen species of PE or PC using this methodology. With a further modification of the gradient and the use of tandem Sperisorb S3W columns we have successfully separated the PE and PC peaks into their various components (data not shown), but have not yet verified peak identities.

The method described in this report is a rapid and accurate procedure for the measurement of lipid with greater sensitivity for phospholipids than previously reported (2). Since certain phospholipids (LPC, PI, and PS) are usually found in the lowest amounts in animal tissue, it is imperative that a detection procedure be sufficiently sensitive to detect low levels of these materials. Our method satisfies this criterion and is also well suited for development as a routine assay to monitor lipid profile changes in many tissues.

Manuscript received 14 September 1989 and in revised form 12 July 1990.

REFERENCES

1. Jungalwala, F. B., J. E. Evans, and R. H. McCluer. 1976. High performance liquid chromatography of phosphatidylcholine and sphingomyelin with detection in the region of 200 nm. *Biochem. J.* **155:** 55-60.
2. Christie, W. W. 1985. Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection. *J. Lipid Res.* **26:** 507-512.
3. Christie, W. W. 1986. Separation of lipid classes by high performance liquid chromatography with the "mass detector." *J. Chromatogr.* **361:** 396-399.
4. Epps, D. E., P. C. Schmid, V. Nataranjan, and H. H. Schmid. 1979. N-acylethanolamine accumulation in infarcted myocardium. *Biochem. Biophys. Res. Commun.* **90:** 628-633.
5. Braughler, J. M. 1985. Lipid peroxidation-induced inhibition of aminobutyric acid uptake in rat brain synaptisomes: protection by glucocorticoids. *J. Neurochem.* **44:** 1282-1288.
6. Folch. J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissue. *J. Biol. Chem.* **226:** 497-509.
7. Wells, M. A., and J. C. Dittmer. 1967. A comprehensive study of the postnatal changes in the concentration of the lipids of developing rat brain. *Biochemistry.* **6:** 3169-3174.