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Short Communication

High-performance liquid chromatographic analysis of free palmitic and stearic acids in cerebrospinal fluid

ILANA ZAMIR* and ELI GRUSHKA

Department of Inorganic and Analytical Chemistry, Hebrew University, Jerusalem (Israel)

and

GABRIEL CIVIDALLI

Pediatric Hematology/Oncology Unit, Hadassah Ein-Karem University Hospital, Jerusalem (Israel)

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ABSTRACT

A relatively simple method for extraction of free fatty acids from cerebrospinal fluid with aminopropyl bonded-phase columns, and the estimation of palmitic acid ($C_{16:0}$) and stearic acid ($C_{18:0}$) concentrations by high-performance liquid chromatographic analysis is described. The values of $C_{16:0}$ and $C_{18:0}$ in patients with non-neurological disorders lie within a narrow range, with a mean (± S.D.) of 4.02 ± 0.33 μg/ml for $C_{16:0}$ and 2.72 ± 0.39 μg/ml for $C_{18:0}$.

INTRODUCTION

Lipids in general, and fatty acids in particular, constitute an essential class of compounds, which are found in physiological fluids such as blood or urine [1]. Chromatographic techniques are among the most popular methods for the analysis of these compounds. There are numerous publications on the analysis of fatty acids in plasma and in urine, but very few dealing with the chromatographic analysis of fatty acdis in cerebrospinal fluid (CSF). Although some reports of the use of gas chromatography (GC) or gas chromatography–mass spectrometry for the analysis of fatty acids in CSF have been published (*e.g.* refs. 2–4), very few papers have described the use of high-performance liquid chromatography (HPLC). This is particularly true for the analysis of free, or unesterified, fatty acids (FFA) in CSF [4,5]. Because changes in the fatty acid composition of the CSF can be anticipated in many inherited and acquired disorders [6,7], the shortage of published studies is surprising: perhaps it is related to the difficulties associated with obtaining CSF samples.

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We present in this paper some preliminary results obtained with an HPLC method that we have developed for the analysis of free (unesterified) palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) acids in CSF. The FFA were isolated from the CSF by solid-phase extraction with aminopropyl bonded-phase columns [8].

The HPLC analysis of FFA is hindered by the lack of a chromophore, which makes sensitive detection very difficult. Therefore, the analysis of FFA requires derivatization with an appropriate chromophore. In the present study, the FFA were derivatized with bromophenacyl bromide (Br-Ph-Br) [9], using 18-crown-6 ether as phase-transfer catalyst [10]. These esters absorb UV radiation strongly at 254 nm, and thus are easy to detect in HPLC [11].

Since CSF from normal individuals could not be obtained for ethical reasons, the analysis was performed on CSF from patients without evident neurological diseases. The samples were drawn from the patients for purposes related to their condition, and the HPLC study was done on samples left over from other analyses.

EXPERIMENTAL

Materials

Aminopropyl bonded-phase plastic columns were purchased from Analytichem International (Harbor City, CA, U.S.A.). The packing material from these columns was removed and added to glass columns. This was done in order to avoid the leaching of impurities from the orginal plastic columns [12]. HPLC-grade hexane, chloroform, 2-propanol, diethyl ether and acetonitrile were obtained from BioLab (Jerusalem, Israel). Standards of FA $C_{16:0}$, $C_{18:0}$ and $C_{21:0}$ were purchased from Fluka (Buchs, Switzerland). Br-Ph-Br and 18-crown-6 ether were obtained from Sigma (St. Louis, MO, U.S.A.).

Patients

CSF was obtained from seven patients with acute leukemia and from two patients with fever and signs of meningeal irritation (lumbar puncture was done to rule out meningitis). After the performance of the routine bacteriological and biochemical tests, the unused CSF was analysed. Three of the patients with acute lymphoblastic leukemia (ALL) and acute non-lymphoblastic leukemia (ANLL) had not received any treatment prior to CSF examination.

Extraction of free fatty acids from CSF

Step 1. Samples of 0.5 ml of CSF from each patient were examined, in duplicate wherever possible. $C_{21:0}$ was added to each sample as an internal standard (0.25 ml of a 5 mg/100 ml solution in chloroform). CSF lipids were extracted six times with equal volumes of chloroform. The pooled extracts were then concentrated under nitrogen to *ca.* 0.5 ml.

Step 2. The concentrates were loaded on activated aminopropyl glass columns,

and sequentially eluted with the following solutions: (1) two 2-ml volumes of chloroform–2-propanol (2:1, v/v): the eluate was discarded; (2) 12 ml of 2% acetic acid in diethyl ether. Acetic acid is needed to release the FFA from the aminopropyl bonded-phase support to allow their complete elution. The eluate, which contains the FFA, was evaporated under vacuum at 83°C for 15 min. The FFA salt was prepared by adding 0.5 ml of chloroform and 1 drop of potassium hydroxide (1% in methanol).

Derivatization reaction

Fatty acid esters were prepared by adding 5 ml of Br-Ph-Br (0.21 g per 100 ml chloroform) and 1 ml of 18-crown-6 ether (0.01 *M*) to the FFA salt. The reaction was carried out in darkness at 60°C for 20 min with stirring. The Br-Ph-Br solution was prepared daily and was kept in the dark at 4°C.

HPLC analysis

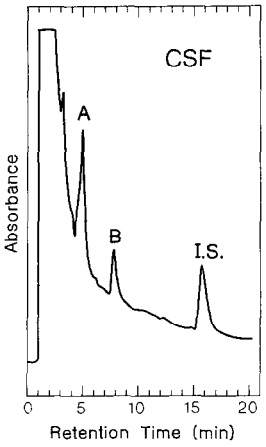
HPLC was performed on a reversed-phase column (LiChrosorb, 12.5 cm × 0.4 cm I.D.; particle size 7 μm) with a guard column (2.5 cm × 0.4 cm I.D.) from E. Merck (Darmstadt, Germany). The mobile phase consisted of 100% acetonitrile. The flow-rate was 2 ml/min. The fatty acid phenacyl esters were detected by UV absorption at 254 nm (Spectra Physics HPLC 8700 and UV detector 8310).

Calculations

To estimate the amounts of FFA in CSF, calibration curves were prepared using standard $C_{16:0}$ and $C_{18:0}$ fatty acid solutions. The acids were dissolved in chloroform, added to 0.5 ml of normal saline, and treated in a similar manner to the CSF samples, including the addition of $C_{21:0}$ as an internal standard. The concentrations of $C_{16:0}$ and $C_{18:0}$ in the CSF were estimated on the basis of the ratios of the areas or heights of the respective peaks to that of the internal standard ($C_{21:0}$), and by comparison with chromatograms of the standard saline solution containing known amounts of the fatty acids.

RESULTS

Fig. 1 shows the chromatogram of $C_{16:0}$ and $C_{18:0}$ phenacyl esters of a typical CSF extract. The peaks were identified by a comparison of the retention times with those of the known fatty acids in the standard solutions (Fig. 2). Confirmation was obtained by adding these standards to CSF samples and analysing them in the same way. In the present chromatographic system, the two fatty acids eluted very close to the very large peak of underivatized reagent, making accurate quantitation difficult. Future studies will attempt to eliminate the great majority of the underivatized reagent so that it will not interfere with the elution of the two acids.



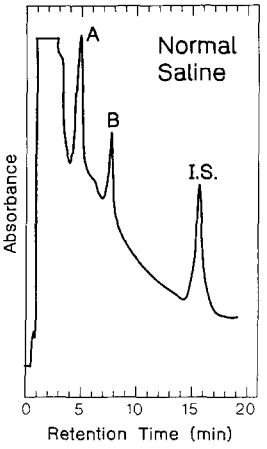


Fig. 1. Chromatogram of FFA in the CSF. Peaks: A = palmitic acid ($C_{16:0}$); B = stearic acid ($C_{18:0}$); I.S. = $C_{21:0}$.

Fig. 2. Chromatogram of standard FA added to normal saline. Peaks: A = palmitic acid ($C_{16:0}$); B = stearic acid ($C_{18:0}$); I.S. = $C_{21:0}$.

TABLE I

CONCENTRATIONS OF PALMITIC AND STEARIC FREE ACIDS IN CSF CALCULATED FROM PEAK AREAS AND PEAK HEIGHTS

Sample No.	Diagnosis[a]	Concentration (μg/ml)			
		Palmitic acid, C_{16}		Stearic acid, C_{18}	
		Height	Area	Height	Area
1	URI, r/o meningitis	4.39	4.12	3.37	3.88
2	URI, r/o meningitis	3.80	4.06	2.60	2.32
3	ANLL, before treatment	3.90	4.16	2.62	2.73
4	ALL, before treatment	3.80	4.02	2.20	2.09
5	ALL, before treatment	3.95	4.40	3.07	3.26
6	ALL, fourth remission	3.45	4.06	2.61	2.26
7	ALL, relapse	4.24	4.73	2.91	2.50
8	ALL, CNS relapse	4.16	4.60	2.94	2.92
9	ALL, sustained remission	4.52	4.02	2.20	2.04
Average value		4.02	4.24	2.72	2.67
S.D.		±0.33	±0.27	±0.39	±0.60

[a] URI = upper respiratory infection; ANLL = acute non-lymphoblastic leukemia; ALL = acute lymphoblastic leukemia.

Using calibration curves, the CSF concentrations of $C_{16:0}$ and $C_{18:0}$ can be estimated. The calibration curve for $C_{16:0}$ was described by the equation: $R = 1.17 - 1.69C$, where R is the $C_{16:0}/C_{21:0}$ peak-height ratio and C is the $C_{16:0}$ concentration. The correlation coefficient was 0.976. The calibration curve for $C_{18:0}$ was $R = 0.97 - 1.07C$. The correlation coefficient was 0.928. The relatively poor fit is due to the fact that the peaks of the acids elute on the tail of the excess derivatization reagent. Nonetheless, these curves allow the estimation of the amounts of the two acids in CSF. Table I shows the amounts of the acids in CSF samples of nine patients without neurological disease. The table gives the amounts calculated both from peak heights and from peak areas. On the whole, the agreement between the two sets of values is very good.

DISCUSSION

Previously published results on long-chain fatty acids in CSF are scanty and extremely variable. The reported methods for their detection and estimation, which use GC or thin-layer chromatography, are cumbersome and have always measured the total (free and esterified) fatty acids [2–4,13,14]. To the best of our knowledge, HPLC has not been applied for the analysis of FFA in CSF, at least not during the past ten years.

The extraction of FFA from CSF by aminopropyl bonded-phase columns is simple and efficient, and it allows the reliable separation of FFA with minimal contamination by triglycerides and other lipids [8]. Thus, the amounts of unesterified $C_{16:0}$ and $C_{18:0}$ can be estimated by HPLC analysis.

The results obtained in the present study, where only two long-chain FFA were analysed, do not show a wide variability. The concentrations of $C_{16:0}$ and $C_{18:0}$ in CSF of patients with various clinical conditions lie in a relatively narrow range, with a mean ($\pm$S.D.) of 4.02 $\pm$ 0.33 μg/ml for $C_{16:0}$ and of 2.72 $\pm$ 0.39 μg/ml for $C_{18:0}$. It is of particular interest to note that, in spite of commonly occurring late CNS sequelae to preventive cranial irradiation of ALL patients [15], no change in the FFA concentrations was observed in their CSF. Application of this method to the measurement of FFA concentrations in the CSF of patients with degenerative and demyelinative diseases of the nervous system, such as multiple sclerosis, may prove valuable for the early detection of these disorders.

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