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Separation and determination of saturated very-long-chain free fatty acids in plasma of patients with adrenoleukodystrophy using solid-phase extraction and high-performance liquid chromatographic analysis

ILANA ZAMIR and ELI GRUSHKA*

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

and

JUAN CHEMKE

Clinical Genetics Unit, Kaplan Hospital, Rehovot (Israel)

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ABSTRACT

An improved method was developed for the isolation of very-long-chain free fatty acids (VLCFFAs) in plasma and their separation and determination by high-performance liquid chromatography (HPLC). The method includes sample clean-up using solid-phase extraction, fluorophoric labelling of the FFAs and reversed-phase HPLC separation. The solid-phase extraction was carried out with aminopropyl-bonded phase columns. The FFAs were then derivatized with 9-anthryldiazomethane (fluorescent) reagent and separated by HPLC on an RP-18 column with methanol as the mobile phase. Using this method, the concentrations of $C_{20:0}$, $C_{22:0}$, $C_{24:0}$ and $C_{26:0}$ were determined in the plasma of five adrenoleukodystrophy (ALD) patients, one obligatory heterozygote, four healthy male volunteers and one child with cerebral leukodystrophy but without any other ALD symptoms. Statistically significant differences were found in the levels of C_{24} and C_{26} and in the ratios C_{24}/C_{22} and C_{26}/C_{22} in ALD patients and in normal controls. The values were higher in patients with X-ALD. This method therefore provides a rapid and accurate procedure for the laboratory confirmation of X-ALD.

INTRODUCTION

Fatty acids play an important role in the central nervous system. Disturbances in their metabolism may cause a variety of genetic disorders. X-Adrenoleukodystrophy (X-ALD) belongs to the lipid storage diseases in which demyelinative changes in the brain and adrenal insufficiency are accompanied by the accumulation of very-long-chain free fatty acids (VLCFFAs) [1–3]. Studies by Singh and co-workers [4,5] have shown that the major defect in X-linked ALD is based on the deficient degradation of VLCFFAs. Hashmi *et al.* [6] suggested that the defect in X-linked ALD is at the level of a deficient ability of peroxisomes to activate VLCFFAs. In a more extensive study, Wanders *et al.* [7] obtained definitive

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evidence that the defect in X-ALD is the deficient peroxisomal activation of VLCFFAs. Therefore, the separation and characterization of fatty acids in body fluids can have a significant value in diagnosing ALD patients.

Reported methods for the determination of the levels of fatty acids, both free and esterified, in complex media such as body fluids and extracts from biological tissues are both cumbersome and time-consuming. Typically these methods involve a pre-analysis treatment, such as extraction and methanolysis, followed by gas chromatographic [8-11] or liquid chromatographic [12] separation.

This paper reports a relatively simple yet accurate and reproducible method of determining FFAs in plasma. The method consists of three main steps: (1) isolation of FFAs from plasma; (2) derivatization of the FFAs for sensitive detection; and (3) reversed-phase liquid chromatographic separation for characterization and quantitation. The isolation of FFAs from plasma was achieved by solid-phase extraction with aminopropyl bonded-phase columns [13,14]. Solid-phase extraction is rapid and can be made highly selective for group separation. In addition, it can be used with relatively small volumes of fluids [14]. Following the isolation from plasma, the FFAs are derivatized to yield a fluorophoric species [15,16] suitable for detection by conventional HPLC detectors. The derivatization step is needed as saturated fatty acids lack a chromophore or a fluorophore and cannot therefore be detected at sufficiently low concentrations. In this work, 9-anthryldiazomethane (ADAM) [17,18] was employed as the fluorescence derivatizing reagent. This reagent converts the carboxyl group of FFA to a highly fluorescent moiety which is suitable for sensitive HPLC analysis. The actual separation, identification and determination FFAs is accomplished using reversed-phase HPLC.

EXPERIMENTAL

Materials

Aminopropyl bonded-phase plastic columns (500 mg of sorbent, about 3 ml capacity, 6 cm length, 0.85 cm I.D. and 10 μm particle size) were purchased from Analytichem International (Harbor City, CA, USA). HPLC-grade hexane, chloroform, propan-2-ol, diethyl ether and acetonitrile were obtained from BioLab (Jerusalem, Israel). Standard fatty acids $C_{20:0}$ (arachidic acid), $C_{22:0}$ (behenic acid), $C_{24:0}$ (tetracosanoic acid), $C_{26:0}$ (hexacosanoic acid) and $C_{27:0}$ (heptacosanoic acid) were purchased from Fluka (Buchs, Switzerland). The ADAM reagent was a gift from Professor N. Nimura (Kitasato University, Tokyo, Japan).

Plasma samples

Heparinized plasma samples were obtained from five X-ALD patients, one obligatory heterozygote (the mother of a patient), four healthy male volunteers (controls) and one patient with cerebral leukodystrophy but without signs of adrenal insufficiency. Standard fatty acids were added to the same control plasma and were used to construct calibration graphs for each analysis.

Instrumentation

The analyses were performed on a Spectra Physics 8700 liquid chromatograph equipped with a 20-μl loop injector and a Chromato-Integrator Model D-2000 (E. Merck, Darmstadt, Germany). The detector was a Model F-1000 variable-wavelength fluorescence spectrophotometer (E. Merck). The flow cell volume was 12 μl.

Procedures

Stock solutions. Standard FFA solutions of $C_{20:0}$, $C_{22:0}$, $C_{24:0}$, $C_{26:0}$ and $C_{27:0}$ (internal standard) were prepared by dissolving 5 mg of FFA in 100 ml of chloroform.

Extraction of free fatty acids from plasma by solid-phase extraction. The isolation of the FFAs was carried out in two stages. (1) Extraction of plasma lipids with chloroform. An internal standard $C_{27:0}$ (25 μl of a 50 mg/l solution in chloroform) was added to 0.5-ml samples of heparinized plasma. Each sample was prepared in duplicate or triplicate. Plasma lipids were extracted six times with equal volumes of chloroform. The pooled extracts were then concentrated under nitrogen to approximately 0.5 ml. (2) Isolation of FFAs on aminopropyl columns. The lipids extract (approximately 0.5 ml) was loaded onto activated aminopropyl columns [12]. The lipid mixture was sequentially eluted with the following solutions: (i) 2 × 2 ml chloroform–propan-2-ol (2:1, v/v); the eluate was discarded; (ii) 12 ml of 2% acetic acid in diethyl ether. The eluate which contains the FFAs was saved and then evaporated at 83°C for 15 min using a Rotavapor evaporator.

Derivatization reaction of free fatty acids. Fatty acid esters were prepared by adding 300 μl of ADAM to the dried fatty acid mixture. ADAM stock solution (1 mg/ml in chloroform) was prepared daily and stored in the dark at 4°C. The derivatization reaction was refluxed, in the dark, at 60°C for 15 min. The mixture was stirred during the reaction.

High-performance liquid chromatographic conditions. The mobile phase consisted of 100% methanol and the flow-rate was 1.5 ml/min. Degassing was carried out with helium before and during use. All organic solvents were filtered through 0.45-μm Type RC-55 membrane filters (Tamar, Jerusalem, Israel). Separations were carried out on a reversed-phase column (LiChrosorb RP-18) of dimensions 12.5 cm × 0.4 cm I.D. The analytical column was protected with a guard column (2.5 cm × 0.4 cm I.D.). Both columns were packed with 5-μm particles (E. Merck). The fluorescence detector was set at 365 nm excitation and at 412 nm emission wavelengths.

Data analysis. The amounts of VLCFFAs in the control plasma were determined using the standard addition method [19]. Known amounts of an acid were dissolved in chloroform and added to 0.5 ml of plasma. A standard addition curve was prepared for each of the FFAs $C_{20:0}$, $C_{22:0}$, $C_{24:0}$ and $C_{26:0}$. To each sample, a constant amount of $C_{27:0}$ was added as an internal standard. The

spiked control plasma samples were then treated in a similar manner to the sample plasma. The standard addition curves were prepared by plotting the area ratio of $C_{27:0}$ to the area of the acid in question *versus* the amount of acid added.

Once the amount of each of the four acids was determined in the control plasma, the standard addition curves were corrected for that amount and redrawn. The corrected plots were used as calibration graphs for the determination of the FFAs in the plasma of patients.

RESULTS AND DISCUSSION

Preparation of the ADAM derivatives

The HPLC analysis of fatty acids is hindered by the lack of a chromophore or fluorophore. Therefore, a derivatization step is needed to obtain high sensitivity. In these studies various chromophores were tested, such as bromophenacyl bromide [20], and fluorophores, such as 4-bromomethyl-7-methoxycoumarin [21], which first required the preparation of the fatty acid salts and then reaction with the phase transfer catalyst 18-crown 6-ether [22]. This procedure was rather cumbersome.

A satisfactory procedure was achieved by using the ADAM fluorescent reagent. The ADAM reagent is particularly easy to use as it does not require the preparation of fatty acid salts to yield fluorescent derivatives. The fluorescent FFA esters give a higher sensitivity, which is required to detect the small amounts of compounds present in biological samples.

Several experimental conditions were examined to find the optimum conditions for making the derivatives. Various ratios of reactants (fatty acids) to reagent (ADAM) were investigated and the optimum ratio was found by peak area to be 1:50 (excess reagent). The role of temperature and reaction time was studied. It was found that heating the reaction mixture at 60°C for 15 min gave the best results. Fig. 1 shows the detector signal intensity, expressed as peak area, as a

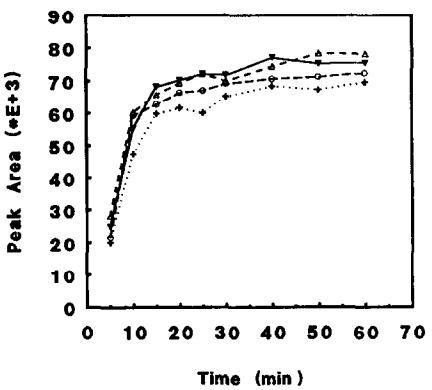

Fig. 1. Peak areas for four fatty acids as a function of the derivatization reaction. Reaction temperature 60°C. (▼) $C_{20:0}$; (△) $C_{22:0}$; (○) $C_{24:0}$; (+) $C_{26:0}$.

TABLE I

REGRESSION DATA AND DETECTION LIMITS FOR SATURATED VLCFFAs DERIVATIZED WITH ADAM

Fatty acid	Slope	Intercept	Regression coefficient	Detection limit (ng/ml)
$C_{20:0}$	1.417	0.8721	0.993	1.25
$C_{22:0}$	0.977	1.2568	0.994	2.50
$C_{24:0}$	0.356	1.2931	0.986	5.00
$C_{26:0}$	0.472	−1.0061	0.988	6.25
$C_{27:0}$	0.348	0.0356	0.989	6.25

function of heating time for the four acids. It is clear that 15 min represents a suitable compromise between the reaction time and completion of the reaction. Recently, a similar derivatization of FFAs has been described [23]. However, the reaction was carried out at room temperature and required 8 h for completion. By carrying out the reaction at 60°C, near completion of the reaction was achieved within 15 min.

To be useful as a quantitation tool, the derivatization process must yield products in direct proportion to the concentration of the acids. In other words, the detector response should be a linear function of the concentrations of acids. In addition, the detection limit of the derivatives must be below the plasma concentration of the fatty acids. The detection response was checked with $C_{20:0}$, $C_{22:0}$, $C_{24:0}$, $C_{26:0}$ and $C_{27:0}$. The detector response was linear in the range 0.03–1.25 μg/ml. The limits of detection for the five acids, as calculated at a signal-to-noise ratio of 3, are given in Table I. Also given in Table I are the slopes, intercepts and correlation coefficients of the linearity plots. Typical plasma values of the above fatty acids are in the range 0.12 − 0.22 μg/ml (see last column in Table II). It can therefore be seen from the detection limits in Table I that this method can be used to monitor the levels of $C_{20:0}$, $C_{22:0}$, $C_{24:0}$ and $C_{26.0}$ in plasma.

TABLE II

REGRESSION DATA FOR STANDARD ADDITION PLOTS OF VLCFFAs RECOVERED FROM SPIKED PLASMA

The last column gives the FFA concentration in the control plasma.

Fatty acid	Slope	Intercept	Regression coefficient	FFA concentration (μg/ml)
$C_{20:0}$	0.4417	−0.0012	0.992	0.222
$C_{22:0}$	0.4824	0.00042	0.995	0.218
$C_{24:0}$	0.4123	0.00055	0.994	0.180
$C_{26:0}$	0.3119	0.0029	0.991	0.127

Fatty acid recoveries from plasma

The analysis of lipophilic compounds in complex media such as plasma involves complicated extraction methods. Some of the published procedures based on liquid–liquid extraction were first tried [8,9]. However, it was found that due to the large volumes of solvents needed for extraction, and the lack of homogeneity, the recovery was relatively low (77–86%) which affected the selectivity and reproducibility of the methods. Therefore it was decided to use solid-phase extraction.

The linearity of the extraction step and the recovery needed to be determined because both of these parameters can be affected by the sample matrix. As normal plasma contains small, but significant, amounts of VLCFFAs, it was decided to use the standard addition method [19]. Using the standard addition method, the linearity of the procedure can be established. In addition, the method allows the determination of the concentration of the indigenous FFAs. A typical standard addition graph is shown in Fig. 2. The figure gives the plot for $C_{26:0}$; similar behaviour was observed for all the acids studied. In Fig. 2, the solid line is the standard addition line. The dashed extension of the solid line is the extrapolation of the standard addition line and the crossing point of the dashed line with the concentration axis yields the amount of acid in the control plasma. Table II gives the slopes, intercepts and correlation coefficients for $C_{20:0}$, $C_{22:0}$, $C_{24:0}$ and $C_{26.0}$ in control plasma. Also shown in Table II are the natural amounts of these acids in the control plasma.

Once the natural amounts of acids are known, they can be corrected by using the following transformation. The equation describing the standard addition line is given by the equation: $AR = a + bS$ where AR is the area ratio, a is the ordinate (y-axis) intercept, b is the slope of the line and S is the amount of standard added. The intercept a is a function of the absorptivity of the acid and

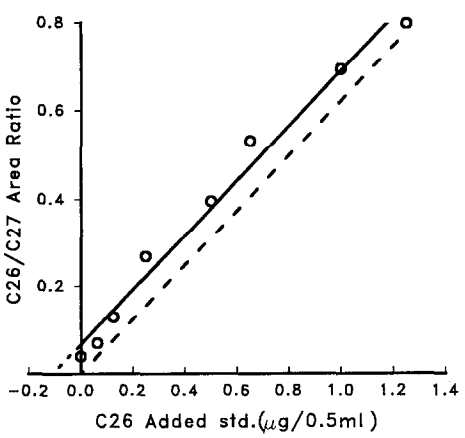

Fig. 2. Standard addition and calibration plots for $C_{26:0}$. The solid line is the standard addition plot; the dashed extension to this is the extrapolation to zero. The broken line is the calibration plot.

its natural concentration in the plasma. Therefore, the transformation: $AR - a = bS = AR'$ gives a new straight line which gives a corrected area ratio, AR', as a function of the concentration of the acid. In Fig. 2 this transformation is given by the broken line parallel to the standard addition line. The result of the above transformation is a calibration plot which can be used to determine the acids in other plasma samples.

The reproducibility of the extraction and derivatization method was evaluated using spiked control plasma. Typical results, expressed in terms of peak area, are shown in Table III. The data in Table III were obtained as follows. Five samples of plasma control were spiked with a solution containing identical amounts of fatty acid standards. The FFAs were isolated using the solid-phase extraction step, derivatized and then injected onto the chromatograph. Each sample was injected five times. The data in each row in the table therefore represent the average (± S.D.) of five replicate injections of a particular plasma sample. Different rows give the average results for different plasma samples. The last row in Table III gives the average (± S.D.) values for the five different samples. An examination of Table III shows that the coefficient of variation of the data, with few exceptions, is less then 5% both for between- and within samples.

The recovery of the acids during the extraction and derivatization steps of the method was between 84 and 90% for each of the acids studied.

Very-long-chain free fatty acids in adrenoleukodystrophy

Fig. 3 shows the HPLC profile of derivatized saturated VLCFFAs in plasma of a normal volunteer (a) and of a patient with X-ALD (b). The peak-area ratios of the fatty acids to the internal standard are clearly higher in the patient with ALD. The large derivatization reagent peak at the beginning of the chromatogram should be noted. Excess reagent can be removed by post-derivatization purification steps, such as thin-layer chromatography. However, it was found that such purification steps result in a marked loss of the fatty acids. Consequent-

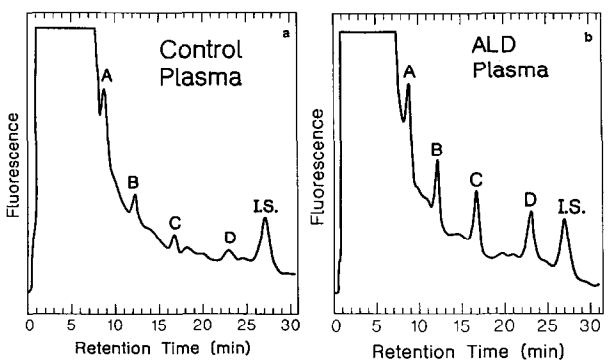

Fig. 3. (a) Chromatogram of FFAs in normal plasma. (b) Chromatogram of FFAs in plasma of ALD patient. Peaks: A = $C_{20:0}$, B = $C_{22:0}$; C = $C_{24:0}$; D = $C_{26:0}$ and I.S. = $C_{27:0}$ (internal standard).

TABLE III

REPRODUCIBILITY, IN TERMS OF PEAK AREAS, OF THE SOLID-PHASE EXTRACTION PROCESS OF VLCFFAs IN SPIKED PLASMA

Values are mean ± S.D. of five injections.

	$C_{20:0}$ (mean ± S.D.)	$C_{22:0}$ (mean ± S.D.)	$C_{24:0}$ (mean ± S.D.)	$C_{26:0}$ (mean ± S.D.)	$C_{27:0}$ (mean ± S.D.)
	34 443 ± 1240	38 948 ± 1520	28 326 ± 943	28 117 ± 1101	27 131 ± 533
	37 968 ± 1753	40 161 ± 1968	29 889 ± 958	28 799 ± 1322	28 822 ± 1200
	34 353 ± 1471	39 753 ± 2345	27 598 ± 821	30 234 ± 483	27 896 ± 1843
	38 293 ± 1317	37 155 ± 2828	30 715 ± 539	27 840 ± 957	27 224 ± 1000
	37 539 ± 988	38 549 ± 3086	28 295 ± 827	26 499 ± 454	28 289 ± 1707
Average ± S.D.	36 519 ± 1955	38 913 ± 1171	28 965 ± 1289	28 298 ± 1367	27 872 ± 999

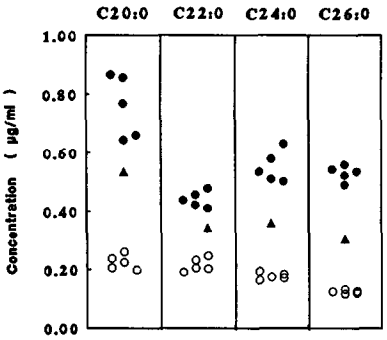

Fig. 4. Schematic representation of FFA concentrations in plasma of (●) ALD patients; (▲) obligatory heterozygote and (○) normal controls.

ly, it was decided not to purify the derivatization products and to use a standard addition method to minimize the baseline effects.

The calibration graphs allow the determination of the amounts of FFAs in the plasma. Table IV and Fig. 4 give the concentrations of the various fatty acids in the plasma of normal volunteers and in five patients with X-ALD. The concentrations of $C_{20:0}$, $C_{22.0}$, $C_{24:0}$ and $C_{26:0}$ are higher in the ALD patients. Table IV also gives the ratios C_{24}/C_{22} and C_{26}/C_{22}, which are used to characterize ALD patients. Fig. 5 shows a schematic representation of these ratios.

The FFA concentrations, in addition to the C_{24}/C_{22} and C_{26}/C_{22} ratios, are clearly higher in the plasma of ALD patients. For C_{24} and C_{26}, the concentrations are about four-fold higher in the ALD patients. The mean of each group in Figs. 4 and 5 is indicated in Table IV, together with its standard deviation. A comparison between the mean concentration in ALD patients and in the normal control, using the Student's *t*-test, indicated that the two groups are different at the 95% confidence level.

FFA values in the normal range were found in the plasma of a two-year-old

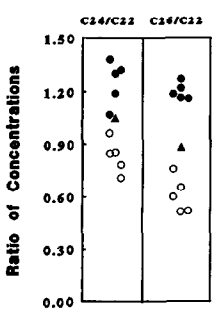

Fig. 5. Schematic representation of C_{24}/C_{22} and C_{26}/C_{22} ratios in plasma of (●) ALD patients, (▲) obligatory heterozygote and (○) normal controls.

TABLE IV

MEANS OF CONCENTRATION OF VLCFFAs IN PLASMA OF ALD PATIENTS, IN HEALTHY MALE CONTROLS AND IN ONE PATIENT WITH CEREBRAL LEUKODYSTROPHY

Also given in the table are S.E.M. values.

Diagnosis	*n*	Concentration (μg/ml)				Ratio	
		$C_{20:0}$	$C_{22:0}$	$C_{24:0}$	$C_{26:0}$	C_{24}/C_{22}	C_{26}/C_{22}
Healthy control	4	0.222 ± 0.014	0.218 ± 0.014	0.180 ± 0.006	0.127 ± 0.003	0.864 ± 0.070	0.613 ± 0.060
ALD	5	0.759 ± 0.047	0.442 ± 0.012	0.553 ± 0.024	0.531 ± 0.011	1.252 ± 0.055	1.202 ± 0.022
ALD heterozygote	1	0.536	0.344	0.360	0.430	1.047	0.884
Cerebral leukodystrophy	1	0.238	0.203	0.172	0.122	0.848	0.601

boy with cerebral leukodystrophy, but without the clinical features of ALD. Intermediate values of FFAs were found in the plasma of the mother of one of one *X*-ALD patient, who is an obligatory heterozygote.

CONCLUSIONS

The method described in this paper combines the use of solid-phase extractions of FFAs in plasma with derivatization with a fluorescing reagent. The method allows a higher recovery of FFAs and it is sensitive, reproducible and relatively simple. Previously reported methods for the detection and estimation of FFAs are cumbersome and usually measure the total free and esterified fatty acids. Most commonly, the total concentrations of VLCFFAs (free and esterified) were measured and analysed by gas chromatography and gas chromatography–mass spectrometry [11,24–26]. The method developed here is relatively simple for the rapid determination of the concentration of VLCFFAs in the plasma of patients with ALD. This method should facilitate the diagnosis of the disease.

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