notes on methodology

One-step rapid extractive methylation of plasma nonesterified fatty acids for gas chromatographic analysis

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Summary The present report describes a one-step method for the derivatization and extraction of nonesterified fatty acids in plasma with subsequent analysis by conventional capillary gas–liquid chromatography or gas–liquid chromatography–mass spectrometry. The procedure requires 200 μl of citrated plasma, dilution with 200 μl of methanol containing a suitable internal standard, and rapid methylation (10 min) with ethereal diazomethane. An aliquot (60%) of the ether layer is subsequently removed, taken to dryness with nitrogen gas, and the residue is dissolved in a small volume of hexane (usually 50 μl) for chromatographic analysis (taking 1 μl for on-column injection). Samples are ready for analysis within 15 min after initial preparation of the plasma. The method has been found to be simple and rapid, providing clean fatty acid profiles. Although the method has been tested with 200 μl of rat and human plasma, it can easily be adapted to a 40 μl plasma sample if the esterified plasma extract is suspended in a smaller volume of hexane and/or a larger aliquot of the extract were to be injected into the gas chromotograph through use of a splitless injector.—**Pace-Asciak, C. R.** One-step rapid extractive methylation of plasma nonesterified fatty acids for gas chromatographic analysis. *J. Lipid Res.* 1989. **30:** 451–454.

Supplementary key words esterification • gas–liquid chromatography–mass spectrometry • internal standard

The specific analysis of nonesterified fatty acids (NEFA) in biological fluids is a difficult and cumbersome procedure. A recent review of the methods used to date has been provided by Lepage and Roy (1). Methods for the estimation of total NEFA include the method of Dole and Meinertz (2), Technicon (3), colorimetry (4–6), titrimetry (7), and enzymatic determinations (8–10). Methods involving the analysis of individual fatty acids have involved some chromatographic step such as GLC (11–15) and high pressure liquid chromatography (16–23), but the procedures for sample preparation have been laborious. A recent report by Lepage and Roy (1) has improved on the method of preparation of samples from plasma for gas–liquid chromatographic analysis and has reported on a simple one-step methylation and extraction procedure that permits analysis of samples within a 45-min period. The procedure reported herein describes an even simpler method through which the direct methylation and extraction of plasma NEFA is carried out in a single 10-min step making possible the analysis by GLC within 15 min of obtaining a plasma sample.

MATERIALS AND METHODS

Diethyl ether was distilled in glass before use. Glass-distilled hexane was purchased from Caledon Labs, Georgetown, Ontario. Diazomethane was prepared in a fume hood by reacting 1 g N'-methyl-N-nitro-N'-nitroso-guanidine (Aldrich) with 10 g potassium hydroxide in 10 ml water, trapping the released diazomethane in 10 ml diethyl ether and distilling the diazomethane in ether. This reagent solution could be stored over potassium hydroxide pellets in a stoppered glass cylinder for about 2 weeks.

Fatty acid standards were purchased as the methyl esters from Chromatographic Specialties, Brockville, Ontario, or as the free acids from Sigma. Pentadecanoic acid (15:0) used as internal standard was purchased as the free acid from Sigma.

Rapid derivatization of plasma NEFA

Blood (1 ml) was withdrawn through a butterfly needle attached to a 1-ml plastic syringe containing 0.1 ml of 3.8% sodium citrate. Blood was withdrawn from the rat (which was lightly anesthetized with ether) through cardiac puncture or from human subjects through venipuncture. The blood was transferred to a plastic tube and centrifuged at 2200 *g* for 15 min to prepare platelet-poor plasma. An aliquot of the plasma (200 μl) was transferred to a glass tube and processed as follows. An equal volume of methanol (200 μl) was added, followed by 12 μg of pentadecanoic acid (4 μl) and gentle mixing. An ethereal diazomethane solution (1 ml) was added and after mixing, the solution was stoppered and stored in a dark container at 22°C for 10 min. Eight hundred μl (corresponding to ca. 60% of the total sample) was transferred to another glass tube and the solvent was taken to complete dryness with a fine stream of nitrogen gas. The residue was dissolved in 50 μl of hexane (or less) and 1 μl (or more) was injected into a gas chromatograph coupled to a mass spectrometer.

Instrumentation

Analysis was performed on a Hewlett-Packard gas chromatograph–mass spectrometer (HP-5970-MSD). The gas

Abbreviations: NEFA, nonesterified fatty acids; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; EI, electron impact; TIC, total ion chromatogram.

chromatograph contained an SP-2331 fused silica capillary column (60 m × 0.25 mm I.D., film thickness 0.20 μm, Supelco) the end of which was inserted into the mass spectrometer source. Hydrogen was used as the carrier gas at a linear velocity of 40 cm/sec. The oven temperature program was similar to that used by Lepage and Roy (1). The oven temperature was maintained at 50°C during sample injection, and was increased to 165°C at 7°C/min. The temperature was then raised at 1.6°C/min to 200°C where it was held for 5 min. The temperature was raised at 0.3°C/min to 204°C at which time the temperature was raised to 220°C at 10°C/min and held there for 10 min at which point the run was terminated and the temperature was recycled to 50°C in preparation for another run. Injector and transfer line were maintained at 230° and 230°C, respectively. Mass spectrometer source pressure was maintained at 4.10^{-5} Torr. Samples were injected on column in 1 μl hexane.

RESULTS

The procedure used in this study for the direct extractive methylation of NEFA from plasma is both simple and expedient. It requires no special equipment other than a well ventilated fume hood for the handling of diazomethane. The procedure results in samples ready for analysis within 15 min of preparation of the plasma and provides samples that are very clean when analyzed by capillary GLC. Typical fatty acid profiles from rat plasma are shown in **Fig. 1**. The profiles are not very different from those recently reported by Lepage and Roy (1) who also described a rapid (45 min) extractive methylation of plasma using an acetyl chloride–methanol mixture. There is one unidentified peak shown in Fig. 1 with a retention time just after arachidonic acid. On an OV-351 column, this contaminant elutes with a retention time between 16:0 and 18:0.

The present method involving the use of diazomethane in diethyl ether ought to be preferred over existing procedures because this reagent is simple to prepare, produces quantitative derivatization, and is quite mild in that it is not known to exert a transesterification reaction. Hence there is little concern about the appearance of fatty acids through transesterification of fatty acids previously esterified to other lipids.

Table 1 shows quantitative data of the major fatty acids observed in five rat plasma samples and three human plasma samples. The rat plasma samples show a total fatty acid content not too different from that published by Lepage and Roy for human plasma (1), although the human plasma total fatty acids in the present study are somewhat lower. Since the procedure of Lepage and Roy depends quite critically on the concentration of reagents used, it is possible that the discrepancy between our values and theirs for the human NEFA may be due to partial hydrolysis through their procedure.

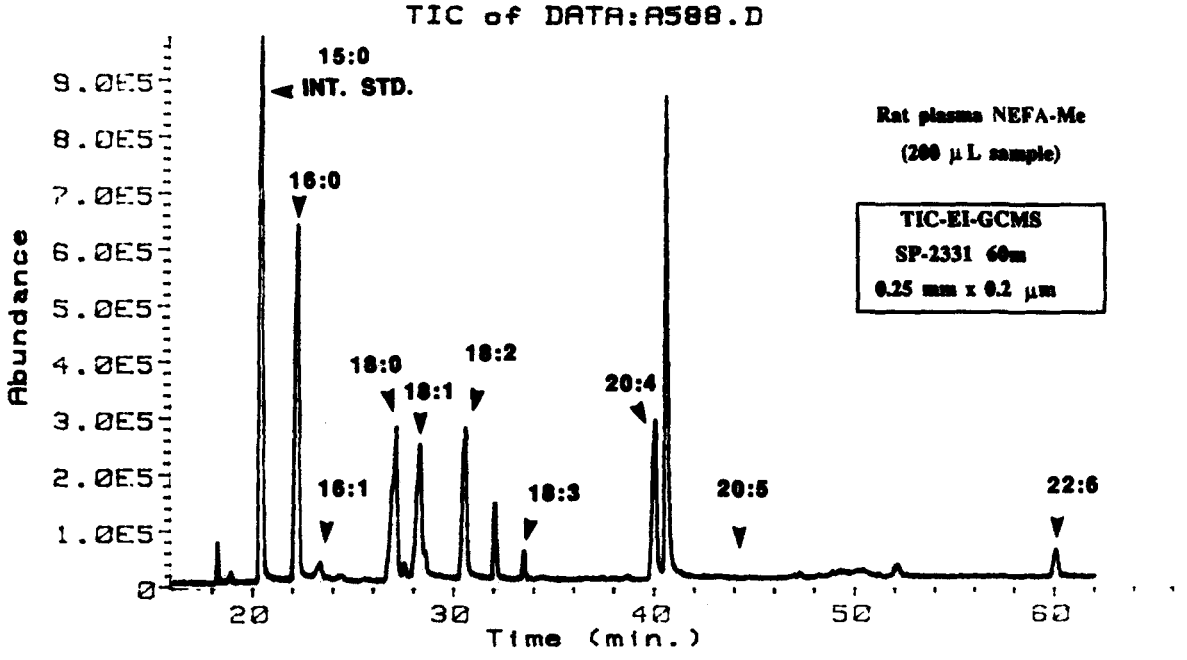

Fig. 1. Total ion chromatogram (TIC) of rat plasma NEFA derivatized by the rapid single-step extractive methylation described in this paper. The GLC-MS was used in the EI mode, employing an SP-2331 fused silica column.

TABLE 1. Plasma NEFA profiles using the rapid extractive methylation procedure described in this report

Fatty Acid	Rat Plasma (n = 5)	Human Plasma (n = 3)
	μg/ml	
16:0	64.2 ± 4.4	48.7 ± 1.6
16:1	5.8 ± 1.2	1.0 ± 0.2
18:0	43.3 ± 3.8	22.5 ± 2.4
18:1	42.6 ± 5.7.	27.8 ± 0.4
18:2	39.6 ± 3.6	20.8 ± 0.9
20:4	23.8 ± 1.9	5.3 ± 1.7
22:6	3.2 ± 1.9	2.0 ± 0.7

Values are expressed as mean ± SEM.

DISCUSSION

A recent report by Lepage and Roy (1) has summarized the deficiencies of available methods for the estimation of NEFA in plasma. These authors have carefully discussed the problems and limitations encountered with these methods in light of their own rapid and efficient method for the extractive methylation of NEFA in plasma. Their method involved the use of methanol solutions of acetyl chloride, care being taken to employ a concentration of acetyl chloride that did not transesterify lipids but would result in quantitative methylation of the NEFA. Although apparently useful, yielding more fatty acids than previous methods, the procedure of Lepage and Roy is not as simple and straightforward as at first it seems to be. Their procedure involves the careful handling of acetyl chloride, stopping of the reaction with potassium carbonate, extraction with hexane, and centrifugation of the samples. The procedure reported herein is much more expedient in that no centrifugation is required, the reagent (diazomethane in diethyl ether) is itself both an extractive and derivatization reagent. No other chemicals are needed to stop the reaction since unreacted diazomethane is blown off within 5 seconds to terminate the reaction. The residue is taken up in as small a volume of hexane as needed for injection into the gas chromatograph. As much as half of the sample can be injected depending on the amount of plasma that is available for analysis. In the present application 200 μl of plasma has been used although the sensitivity of the instrumentation is such that 40 μl of plasma could easily have been analyzed. The method is highly suited for the investigation of the effects of drugs on plasma NEFA in small animals or human neonates where limited amounts of plasma are available for sequential sampling or in perfusates of tissues.

The procedure described herein provides a single step, single reagent, rapid (10 min), quantitative and simpler method available so far for the extractive methylation of NEFA in plasma. Samples can be ready for analysis within 15 min of the availability of a plasma sample or within 30 min from the availability of a blood sample.

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