papers and notes on methodology

A rapid, simple, and sensitive procedure for the determination of free fatty acids in plasma using glass capillary column gas–liquid chromatography

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Abstract A rapid and sensitive method for the analysis of plasma free fatty acids with glass capillary column gas–liquid chromatography and flame ionization detection is described. The plasma sample, together with n-pentadecanoic acid as an internal standard, was treated with 2,2-dimethoxypropane and hydrochloric acid. 2,2-Dimethoxypropane serves as a water scavenger, deproteinizing agent, and as a methylating agent. Under the assay conditions, only free fatty acids were converted to their methyl esters; esterified fatty acids, such as those in triglycerides and phospholipids, were not significantly transmethylated. This advantage eliminated the need for thin-layer chromatography for the separation of free and esterified fatty acids. The methyl esters of fatty acids were then extracted into isooctane and analyzed with a 10-meter glass capillary column coated with SP-2100. Splitless mode of injection was used to increase the sensitivity. Only 20 μl or less of plasma was required for analysis. The coefficient of variation was 4.6%, which was better than the conventional gas–liquid chromatographic methods. These latter methods require 20 to 50 times larger samples, as compared with the present assay. This method is suitable for the measurement of both total free fatty acids and individual fatty acid patterns in small plasma samples.—**Tserng, K-Y., R. M. Kliegman, E-L. Miettinen, and S. C. Kalhan.** A rapid, simple, and sensitive procedure for the determination of free fatty acids in plasma using glass capillary column gas-liquid chromatography. *J. Lipid Res.* 1981. **22:** 852–858.

Supplementary key words nonesterified fatty acids · dimethoxypropane methylation

Conventionally, the determination of total free fatty acids in plasma has been done by either titrimetric or colorimetric methods (1). Both these methods have the disadvantage of being nonspecific. A number of normal plasma constituents, such as phospholipids and organic acids, are known to cause interference. This is especially true in the case of pregnancy, diabetes, and nephrotic syndrome, where the plasma concentration of these interfering substances is increased (2). Besides, these methods lack sensitivity and require at least 100 μl of plasma for analysis, although a recent publication has reported the use of only 40 μl of plasma (3). The newer gas–liquid chromatographic methods are more specific and permit measurement of both total free fatty acids and individual fatty acid components simultaneously (2, 4, 5). Unfortunately, current methods, which involve solvent extraction, purification, derivatization, and gas–liquid chromatography, are lengthy and cumbersome. Besides, their sensitivity is worse than that of the previously mentioned methods. In general, a plasma sample size of 0.5–2 ml is required. In our study of fuel metabolism among newborn infants, a sensitive micro-method for the determination of plasma free fatty acids was required because of the limitation of blood sampling. In addition, this method should be able to determine the individual fatty acid pattern for maximal information. The large number of samples generated from these studies also

Abbreviations: DMP, 2,2-dimethoxypropane; GLC, gas–liquid chromatography; 14:0, myristic acid; 15:0, pentadecanoic acid; 16:0 palmitic acid; 17:0 heptadecanoic acid; 16:1, palmitoleic acid; 18:0 stearic acid; 18:1, oleic acid; 18:2 linoleic acid; 18:3, linolenic acid.

called for a rapid procedure. None of the currently available methods met these requirements. Therefore, a simple and sensitive micro-method for the determination of free fatty acids using glass capillary column gas–liquid chromatography was developed.

MATERIALS AND METHODS

Reagents

Dimethoxypropane (DMP), purchased from Aldrich Chemical Co., Milwaukee, WI, was purified by distillation under vacuum at 40–50°C and stored at room temperature. Isooctane (glass-distilled grade) was supplied by Burdick and Jackson Lab., Muskegon, MI. All other reagents were reagent grade and were used without further purification.

Pentadecanoic acid and heptadecanoic acid were purchased from Supelco, Bellefonte, PA. All glassware was rinsed three times with methylene chloride (0.5 ml) and air dried.

Standard mixture for gas–liquid chromatograph calibration

This mixture was prepared by mixing methyl pentadecanoate (Supelco) and fatty acid methyl ester standard mixture RM-6 (Supelco). It was then diluted with isooctane. The final dilution contained the following fatty acids in 1 μl: methyl pentadecanoate, 12.6 ng; methyl myristate, 0.8 ng; methyl palmitoleate, 1.3 ng; methyl palmitate, 12.0 ng; methyl linoleate, 2.8 ng; methyl linolenate, 1.2 ng; methyl oleate, 16.5 ng; and methyl stearate, 5.6 ng.

Isolation and derivatization

In a disposable glass culture tube (12 × 75 mm), 50 μl of plasma was mixed with 25 μl of internal standard solution containing 3.01 μg of pentadecanoic acid in methanol, 1 ml of DMP, and 20 μl of concentrated hydrochloric acid. It was then capped and kept at room temperature for 15 min. Pyridine, 10 μl, was added to stop the reaction, and the mixture was concentrated in a stream of air to about 100 μl. It was then diluted with 0.5 ml of water. The aqueous mixture was extracted with 0.5 ml of isooctane. After centrifugation to separate the layers, the isooctane layer was transferred (as much as possible) to a 1-ml serum vial (Wheaton Scientific Co., Millville, NJ), and was evaporated in a stream of air to dryness. The vial was capped with a Teflon-lined serum cap (Fisher Scientific Co., Fairlawn, NJ). Isooctane (30–50 μl) was then added with a microsyringe (Hamilton Co., Reno, NV) through the cap. After mixing thoroughly, an aliquot (0.5 μl) was injected into the gas chromatograph.

Gas–liquid chromatography

A Hewlett-Packard 5840A model gas chromatograph (Hewlett-Packard Co., Avondale, PA) equipped with a 18835B capillary inlet system and a flame ionization detector was used. The column was a wall-coated glass capillary column (0.25 mm × 10 m), coated with SP-2100 (J & W Scientific Company). The column was connected to the injection port and the detector with a rubber O-ring after the end was straightened and deactivated with carbowax 20M. A splitless mode of injection was used. The solvent-purging valve was activated 0.7 min after injection. Helium was used as carrier gas at a flow rate of 1.5 ml/min. The flow rate of the make-up gas (nitrogen) for the detector was 35 ml/min. The injection port temperature was 230°C, the detector was 300°C, and the auxiliary temperature was 250°C. The column temperature was held at 100°C during the injection. It was then raised at the rate of 30°C per minute to 190°C; held at 190°C for 8 min and then raised again to 230°C at the rate of 30°C per minute. The column temperature was maintained at 230°C for an additional 3 min, and cooled down to 100°C for the next analysis.

The following retention times (min) were observed for the individual fatty acids: 14:0, 3.23; 15:0, 3.80; 16:1, 4.33; 16:0, 4.54; 18:2 and 18:3, 6.44; 18:1, 6:59; and 18:0, 7.11.

Each day before analysis, the gas chromatograph was calibrated using a standard mixture of fatty acids. Their retention times and amounts (expressed either as μg or μmole) were entered through the keyboard using the internal standard method program of the HP5840A gas chromatograph. The amount of internal standard and the dilution factor for each series of samples was then entered. Total free fatty acid concentration was obtained by the sum of individual fatty acids.

Determination of optimal conditions

In order to determine the optimal conditions necessary for methylating free fatty acids without transmethylating triglycerides and phospholipids, pooled plasma samples were spiked with pentadecanoic acid and analyzed according to the present method, with different amounts of hydrochloric acid and at varying reaction times. After terminating the reaction by the addition of pyridine, another internal standard, methyl heptadecanoate, was added. The samples were then worked up as described above.

The second internal standard (17:0) served as an

indicator of complete methylation of free fatty acids. The area ratio between 15:0 and 17:0 as well as the area ratio between individual plasma fatty acids and 17:0 were determined. Since natural 15:0 is present only in negligible amounts in human plasma, the area ratio between 15:0 and 17:0 should become constant upon quantitative conversion. However, the ratio between other plasma fatty acids and 17:0 would reach a constant value if free fatty acids only were methylated. The area ratio between plasma fatty acids with 17:0 and 15:0 would continue to rise if fatty acids from sources other than free fatty acids were methylated.

Derivatization with diazomethane

Plasma, 50 μl, was mixed with 25 μl of internal standard solution and 1 ml of methanol. After centrifugation, the supernatant was transferred into another glass culture tube, evaporated to dryness with a stream of air, and the fatty acids were methylated by adding diazomethane in ether. The diazomethane was freshly generated from N-methyl-N-nitro-N-nitrosoguanidine (Aldrich Chemical Co.). The ethereal solution was kept at room temperature for 2 min and then evaporated to dryness. The residue was partitioned between 0.5 ml of water and 0.5 ml of isooctane. The isooctane layer was further treated as described above.

Colorimetric method for the determination of total free fatty acids

The micro-method described by Novak was used (6). Plasma samples, 50 μl, were used for the analysis.

RESULTS AND DISCUSSION

Gas–liquid chromatographic determination of free fatty acids has a number of advantages over the classic titrimetric or colorimetric methods. The free fatty acids are isolated from the biological fluid, separated from other fatty acid-containing components, derivatized to a volatile form, and then analyzed by GLC.

The isolation of free fatty acids from plasma is usually achieved by solvent extraction adapted from either "Dole lipid extraction" or "Folch lipid extraction" (7, 8). The different modifications of solvent mixtures were evaluated and commented on by Regouw et al. (1). An alcohol, such as methanol or 2-propanol, is an essential part of the solvent mixture. It serves as a protein precipitating agent required to free the unesterified fatty acids from serum albumin. Although these solvents are claimed to be effective, the sample recovery generally has been variable. Furthermore, the recovery of different fatty acids might be different as has been described by Gerber, Barnes, and Nies (9). In these procedures isolated free fatty acids are separated from interfering triglycerides and phospholipids by thin-layer chromatography, which increases the time required for analysis. In addition, thin-layer chromatography introduces interference peaks from the silica gel scrapings (4). The popular rapid purification method of Hagenfeldt (5) (see also 10 and 11) using alkali-methanol extraction to separate free fatty acids from triglycerides hydrolyzed significant amounts of triglycerides or phospholipids that were co-extracted with free fatty acids. As a result, falsely high free fatty acid levels were obtained. The conversion of free fatty acids to the volatile methyl ester for GLC analysis is usually achieved by boron trifluoride-methanol, diazomethane, or hydrochloric acid-methanol methods. All of these methods have their disadvantages. Diazomethane is inconvenient to use, besides the fact that it reacts with unsaturated fatty acids to form pyrazoline products (12). Hydrochloric acid-methanol has the disadvantage of slow reaction and non-quantitative conversion. The use of boron trifluoride-methanol requires prior separation of free fatty acids from triglycerides and phospholipids, which are known to be transmethylated with methanol to form fatty acid methyl esters, under the assay conditions.

To overcome the difficulties discussed above, the present procedure combined the isolation, purification, and derivatization into one step. Dimethoxypropane was used to achieve these goals. Dimethoxypropane is a water scavenger, therefore it facilitates the methylation of fatty acids (13). Furthermore, methanol produced from DMP upon its hydrolysis, serves as a protein precipitating agent and a source of methyl groups. The greatest advantage is that DMP converts free fatty acids to their methyl esters but is not reactive enough to transmethylate esterified fatty acids in triglycerides and phospholipids upon brief contact.

Plasma with the internal standard, pentadecanoic acid, was mixed with dimethoxypropane. Upon addition of hydrochloric acid, the plasma proteins were precipitated immediately and the methylation of free fatty acids was completed within 15 min. The optimal conditions required for methylation of free fatty acids only, without disturbing triglycerides and phospholipids, were 20 μl of concentrated hydrochloric acid in 1 ml of DMP kept at an ambient temperature for 15 min. Under these conditions, the methylation of free fatty acids was complete and the transmethylation with triglyceride and phospholipid was

TABLE 1. Comparison of plasma free fatty acids with two methods[a]

Fatty Acids	Plasma Levels: DMP Method (n = 3)	Plasma Levels: CH_2N_2 Method (n = 4)[c]	*t*-Test[d]
	(μg/ml)[b]		
14:0	3.14 ± 0.66	2.85 ± 0.65	N.S.
16:0	50.84 ± 2.71	45.76 ± 6.77	N.S.
16:1	4.85 ± 0.96	5.62 ± 1.12	N.S.
18:0	23.39 ± 2.09	21.83 ± 2.36	N.S.
18:1	54.47 ± 3.60	57.82 ± 6.18	N.S.
18:2	63.22 ± 1.50	46.77 ± 5.51	$P = 0.002$
Total	199.91 ± 8.80	179.92 ± 16.65	$P = 0.04$

[a] Plasma of a fasting adult man was used for this study.
[b] Mean ± S.D.
[c] Diazomethane methylation.
[d] Grouped *t*-test; N.S. = not significant.

negligible. Prolonged reaction and stronger acid concentrations increased the transmethylation slowly, but significantly. When smaller amounts of plasma were used, the amount of reagents had to be decreased proportionally. To check further that the present procedure measured the true, uninflated free fatty acid value, an experiment was done to compare this procedure with the diazomethane method (**Table 1**). Diazomethane reacts only with free acids, but not the esterified acids. As shown, the two methods gave similar results. The only difference was the peak contributed from 18:2 and 18:3. The diazomethane method showed significantly lower results than the DMP method. This difference was readily explained by the high reactivity of diazomethane with polyunsaturated fatty acids to form pyrazoline derivatives (12). The observation that this peak diminished progressively upon prolonged contact with diazomethane tended to support this explanation. Thus, it can be concluded that no significant amount of free fatty acid methyl esters was derived from transmethylation in the present procedure.

The extraction of fatty acids with isooctane after their conversion to methyl esters increased recovery and also eliminated the possibility of differential extraction among different fatty acids. This was due to the fact that methyl esters are highly lipophilic. Furthermore, the ester form also eliminated the influence of pH and ionic strength over ionization of different fatty acids.

High resolution capillary column gas–liquid chromatography with flame ionization detection was used for quantitation. Besides the increase in sensitivity due to narrow peaks, this technique also has the advantage of separating interfering plasma peaks from fatty acids. Packed columns, such as diethylene glycol succinate or cyanopropylsilicone (SP-2330, Supelco), were also evaluated. With these columns, the interfering peaks co-eluted with the internal standards (either pentadecanoic acid or heptadecanoic acid) and other fatty acids, and resulted in variable and misleading results.

The stable non-polar methylsilicone (SE-30 or SP-2100) phase was used in the present method. A 10-meter column was adequate to separate all fatty acids

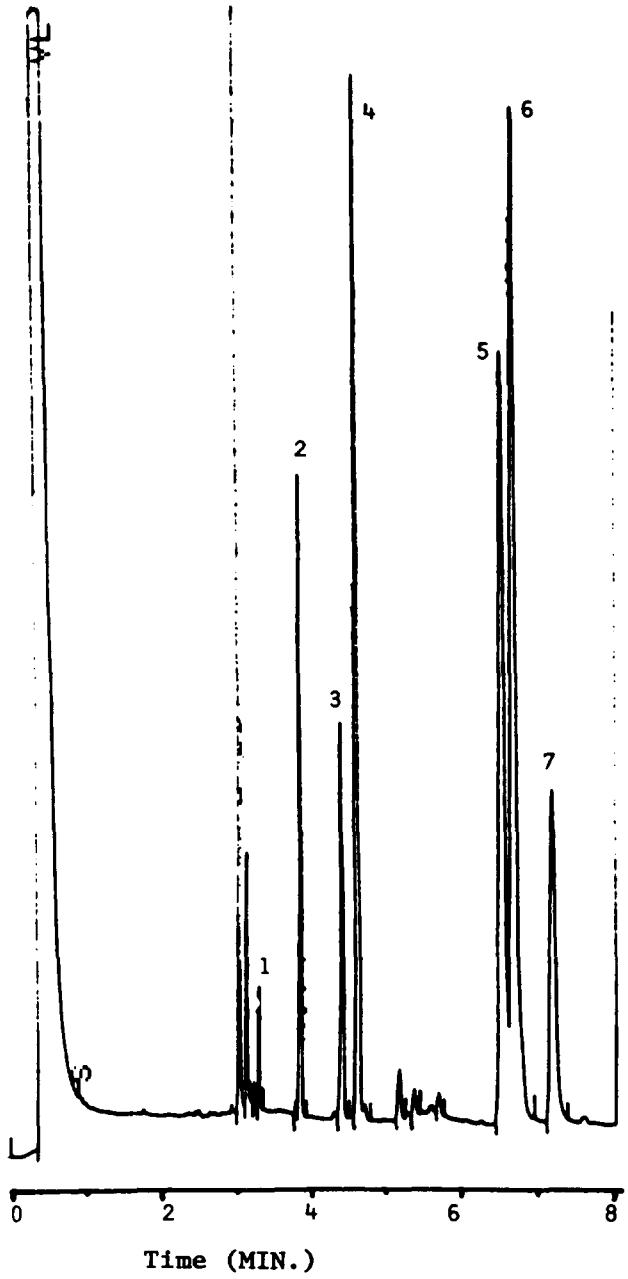

Fig. 1. Gas chromatogram of plasma free fatty acids from a fasting diabetic pregnant dog. The fatty acids are: 1, 14:0; 2, 15:0; 3, 16:1; 4, 16:0; 5, 18:2; 6, 18:1; 7, 18:0.

TABLE 2. Precision of DMP-capillary GLC measurement of free fatty acids[a]

Fatty Acids	Plasma Concentrations					
	GLC Response[b] (n = 6)	C.V.	Intraassay[c] (n = 6)	C.V.	Interassay[d] (n = 9)	C.V.
	μg/ml	%	μg/ml	%	μg/ml	%
C14:0	2.66 ± 0.08[e]	3.01	3.08 ± 0.37	12.01	3.59 ± 0.81	22.56
C16:1	4.77 ± 0.12	2.52	4.88 ± 0.18	3.69	5.17 ± 0.27	5.22
C16:0	50.91 ± 0.39	0.77	49.29 ± 2.02	4.10	54.80 ± 1.89	3.46
C18:2	57.21 ± 0.65	1.14	56.87 ± 2.85	5.01	71.97 ± 3.75	5.21
C18:1	58.67 ± 0.74	1.26	58.18 ± 4.37	7.51	64.95 ± 4.24	6.53
C18:0	23.61 ± 1.70	7.20	20.61 ± 2.74	13.29	26.72 ± 1.44	5.39
Total	197.82 ± 2.78	1.41	193.63 ± 10.07[f]	5.20	227.84 ± 9.11[f]	4.00

[a] Plasma from a fasting (16 hr) male adult was used for study. Fresh plasma was assayed immediately for the determination of GLC response and intraassay variations. Aliquots of plasma (50 μl each) were pipetted into individual tubes and kept frozen at −20 C. The frozen plasmas were used for interassay measurement for up to 4 days.
[b] The variations due to injecting one sample for six times (instrument variation).
[c] Obtained by analyzing six samples in the same run.
[d] Data pooled in different runs and different days.
[e] Mean ± S.D.
[f] $P = 0.001$ with grouped t-test of unequal variance.
C.V., coefficient of variance.

within 10 min. The only exceptions were linoleic and linolenic acids, which eluted together. However, since linolenic acid is present only in trace quantities in the plasma free fatty acid fraction, its concurrent elution with linoleic acid did not present a major drawback. Theoretically, the sample size required can be reduced to 1 μl. In practice, however, a sample size of 20 to 50 μl was found to be more convenient to work with. A typical chromatogram from the analysis of plasma free fatty acids is shown in **Fig. 1.** The linearity of the detector response to different fatty acids was established using authentic samples.

The precision of the present method is displayed in **Table 2.** The coefficient of variation, due to the instrument itself, was 1.41%. There were no statistical differences in the intra-assay and inter-assay variations. The average coefficient of variation was 4.6%.

The data in Table 2 also show that the concentration of free fatty acids in frozen plasma samples (column 6) was higher than that in the fresh samples (column 4), probably as a result of hydrolysis of the esterified fatty acids due to freezing and thawing. The controversy over the stability of free fatty acid patterns in frozen plasma remains (14, 15). Trichopoulou, Kalaidzidou, and Kalandidi (14) observed increases in free fatty acids in frozen plasma within 24 hr. On the other hand, Rogiers (15) observed no change in 10 days. Our data show a rapid hydrolysis initially and then no further change over several days. This is similar to the observation on erythro-

TABLE 3. Plasma free fatty acids determined by DMP-capillary GLC procedure

Population	Subjects	Plasma Free Fatty Acids						
		C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	Total
					μg/ml			
Human premature infants[a]	1	1.85	11.48	1.10	6.62	6.19	3.96	31.2
	2	5.05	18.43	3.65	4.01	13.00	3.28	47.4
	3	5.55	23.72	5.40	4.72	15.51	13.00	67.90
	4	1.80	25.67	4.76	6.00	15.87	10.31	64.41
Dog pups[b] (newborn)	pup 1	3.62	27.71	13.14	7.08	11.43	17.37	80.35
	pup 2	2.77	25.89	8.73	7.17	8.86	14.25	53.42
	pup 3	3.11	30.46	11.49	10.51	13.82	13.75	83.14
	pup 4	1.85	30.23	8.14	10.42	9.97	10.63	71.24

[a] 50 μl of plasma sample.
[b] 20 μl of plasma sample.

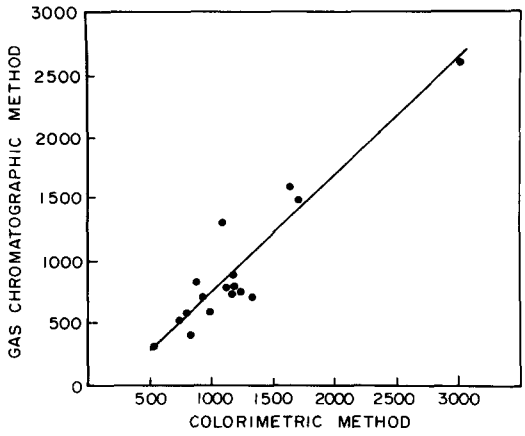

Fig. 2. Correlation between the total free fatty acids results measured by the present method and by the colorimetric method of Novak (6). The total free fatty acid is expressed as μg/ml. The regression equation is Y = −205.32 + 0.93 X (r = 0.8807).

mycin estolate, the propyl ester of erythromycin, in plasma (16).

We have used this method for the analysis of plasma free fatty acids in human premature infants and newborn dogs. Results from the representative plasma free fatty acid analyses are listed in **Table 3.** Free fatty acid patterns and total free fatty acids values are in agreement with those reported in the literature (17, 18).

To compare the DMP method with the colorimetric methods, plasma samples were analyzed by the present method and by the colorimetric method of Novak (6). The results are shown in **Fig. 2.** The values by the present method were lower than those by the colorimetric method. The same relationship has been observed by McDonald-Gibson and Young (2) in comparing their GLC method with the colorimetric method. Nevertheless, the results of the two methods were highly correlated (r = 0.8807). Since the fatty acids analyzed (by the GLC methods) account for more than 90% of the total free fatty acids (11), the lower results cannot be explained by the unaccounted free fatty acids alone (1). The difference could be due to the nonspecific nature of the colorimetric methods.

CONCLUSION

A simple, rapid, and sensitive glass capillary column gas–liquid chromatographic method for the analysis of plasma free fatty acids is described. This method is highly reproducible (coefficient of variation, 4.6%). The sample size required is only 20 μl and can be reduced further. Twenty samples require only about 1 hr to prepare until ready for the GLC analysis, which is the rate-limiting step in the entire procedure. Each GLC run takes about 16 min, including time used for cleaning the column at higher temperatures and the time required for cooling down to the starting temperature for the next analysis. With the incorporation of an automatic sample injector, such as the one described by Jaeger, Klör, and Ditschuneit (19), this drawback can be overcome and the procedure can be rendered even more appealing for routine clinical analysis.

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