Journal of Chromatography, 100 (1974) 35–42

CHROM. 7683

PREPARATION OF METHYL ESTERS FROM THE SAPONIFIABLE FATTY ACIDS IN SMALL BIOLOGICAL SPECIMENS FOR GAS–LIQUID CHROMATOGRAPHIC ANALYSIS

JOSEPH MacGEE and KENNETH G. ALLEN

Basic Science Laboratory, Veterans Administration Hospital, 3200 Vine Street, Cincinnati, Ohio 45220, and the Departments of Biological Chemistry and Medicine, College of Medicine, University of Cincinnati, Cincinnati, Ohio (U.S.A.)

(Received May 28th, 1974)

SUMMARY

Tissue (3–5 mg) or serum (5–100 μl) is hydrolyzed with alkali, acidified and extracted with hexane. The fatty acids are extracted from the hexane into a small volume of trimethyl(α,α,α-trifluoro-*m*-tolyl)ammonium hydroxide. Injection of the resultant quaternary ammonium salts together with methyl propionate into a gas chromatograph yields the fatty acid methyl esters. Preparation of the sample requires less than 45 min and less than 10 min of the analyst's time. The results are comparable with those obtained by conventional methods with larger samples.

INTRODUCTION

The preparation of fatty acid methyl esters for the gas–liquid chromatographic determination of the fatty acids in a biological specimen can be performed by a well trained analyst in a well equipped chemical laboratory. One of the best procedures for such an analysis involves extraction of the lipids with a mixture of chloroform and methanol[1] followed by alkali-catalyzed methanolysis of the naturally occurring esters[2] and then acid-catalyzed methylation of any free acids[3]. The major disadvantages of such an approach are the time required, much of the analyst's attention, relatively large volumes of solvents, back-washing, drying, evaporating, transferring of extracts and reaction products, and preparation and storage of anhydrous reagents. In addition, when small amounts of tissue are to be analyzed, irreversible adsorption of the lipids, acids or esters can occur on the surface of the glassware and the esters can be lost on prolonged evaporation of the various solutions. In spite of these difficulties, reliable results are generated in many laboratories, but the best results are only obtained in those laboratories where much care is taken.

A procedure that is expected to replace any of the conventional methods must offer real advantages in addition to good accuracy and precision. The method described in this report meets these requirements.

MATERIAL AND METHODS

Reagents

Methanolic potassium hydroxide was prepared by dissolving 15 g of potassium hydroxide pellets (certified A.C.S., Fisher Scientific, Pittsburgh, Pa., U.S.A.) in methanol, cooling to room temperature, and dilution to 100 ml with methanol. Insoluble potassium carbonate was removed by centrifugation.

Phosphoric acid (1 *M*) was prepared by adding 13.6 ml of phosphoric acid (reagent grade, 85%, Merck, Rahway, N. J., U.S.A.) to about 150 ml of distilled water. After cooling, the volume was diluted to 200 ml with distilled water.

The hexane used was certified A.C.S. hexanes (Fisher Scientific).

Trimethyl(*α,α,α*-trifluoro-*m*-tolyl)ammonium iodide (TMTFTI) was prepared as follows. A mixture of 10 ml of *α,α,α*-trifluoro-*m*-toluidine (practical grade, Eastman Organic, Rochester, N.Y., U.S.A.) and 20 ml of methyl iodide (certified grade, Fisher Scientific) in a stoppered 250-ml erlenmeyer flask was stored for 24 h in the dark at room temperature. A 50-ml volume of methanol and two boiling stones were added to the crystals and the suspension was heated in a vent hood until the vapors in the neck of the flask reached a temperature between 60 and 65°. The flask was immersed in an ice-bath for 10 min, and the product was then collected on a buchner funnel. The crystals were washed with a small volume of ice-cold methanol on the funnel, and suction was continued until the excess of solvent was removed. The damp product was transferred back into the erlenmeyer flask and recrystallized from 25 ml of methanol. The solution was chilled, collected and washed as before. On vacuum drying at 50°, typical yields between 1.3 and 1.8 g were obtained.

Aqueous trimethyl(*α,α,α*-trifluoro-*m*-tolyl)ammonium hydroxide (TMTFTH) (0.5 *M*) was prepared by mixing 0.66 g of TMTFTI (from above), 0.35 g of silver oxide (purified, Fisher Scientific), and 4.0 ml of distilled water together in a test-tube until all the TMTFTI had dissolved. After centrifugation at 600 *g* for 1 min, an aliquot of the clear supernatant fluid was diluted ten-fold with distilled water and tested for iodide with 0.1 *M* silver nitrate in 6 *M* nitric acid. If a positive halide test resulted, the agitation, centrifugation and testing procedures were repeated until no iodide could be detected in the reagent solution. The reagent solution was stored in a refrigerator.

Methyl propionate–methanol (1:2) was prepared by mixing one volume of methyl propionate (Eastman Organic), which had been treated with anhydrous sodium carbonate, with two volumes of methanol.

Instrumentation

Gas–liquid chromatographic analyses were performed on a Perkin-Elmer F11 MK II dual flame instrument with 12 ft. × 1/4 in. (3 mm I.D.) glass columns with removable glass inserts in the injection ports. The columns were packed with pretested 10% EGSS-X on Gas-Chrom P, 100–120 mesh (Applied Science Labs., State College, Pa., U.S.A., Lot SP-1076). A 0–5 mV recorder was used. The injection ports were maintained at 240° and the columns at 180°. The carrier gas was nitrogen at a flow-rate of 38 ml/min.

Procedures

Total saponifiable fatty acids from 100-μl serum samples. The serum sample

was mixed with 0.5 ml of methanolic potassium hydroxide in a 15-ml glass-stoppered centrifuge tube and heated for 30 min at 65° in a Temp-block heater. After cooling with tap water, 0.7 ml of 1 *M* phosphoric acid was added, the sample was mixed briefly on a vortex mixer and again cooled. A 5-ml volume of hexane was added and the tube was shaken vigorously by hand for 1 min and then centrifuged at 600 *g* for 1 min. Nearly all of the hexane extract was transferred into a 5-ml glass-stoppered conical centrifuge tube (Pyrex No. 8061) with a disposable pasteur pipette. It was not necessary to transfer all the hexane, but care was taken to avoid transferring any of the aqueous phase. A 10-μl volume of the aqueous TMTFTH reagent was used to extract the fatty acids from the hexane. The contents of the tube were shaken and centrifuged as above. An aliquot of 1 μl of the TMTFTH extract (lower phase) was sandwiched between two plugs of the methanolic methyl propionate mixture in the injection syringe in the following way. A 10-μl syringe was pre-wetted with the 1:2 methyl propionate–methanol, the needle was wiped, 1 μl of the TMTFTH extract was drawn

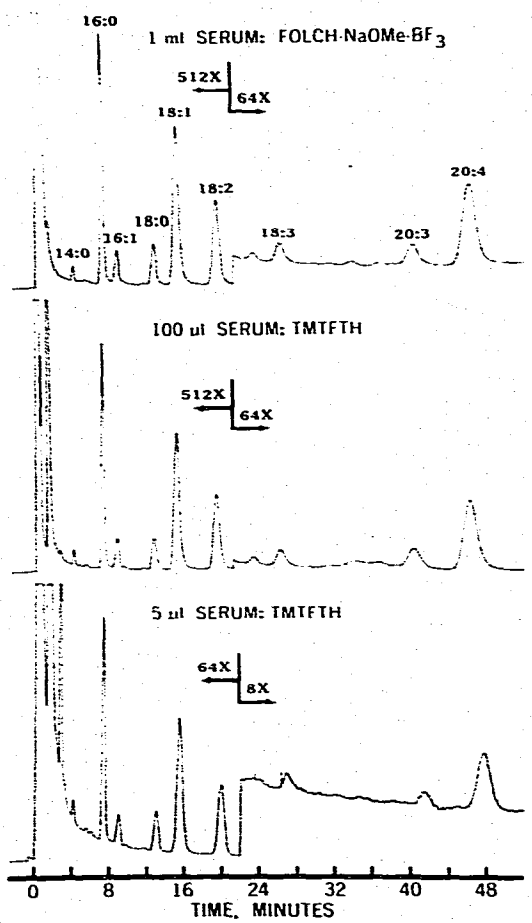

Fig. 1. Gas–liquid chromatography of fatty acid methyl esters derived from pooled human blood serum. The upper curve shows the elution pattern obtained by a conventional procedure (see text). The lower patterns were obtained by the new procedures described in this paper.

into the syringe, the needle was again wiped, and 0.5 μl of the methyl propionate solution was taken into the syringe. Drawing the plunger of the syringe back to the 5-μl mark and then depressing it to the 3-μl mark brings about sufficient mixing. The sample was finally injected into the chromatographic unit. The results are shown in Fig. 1.

Free fatty acids from serum. This procedure was similar to the one for the total fatty acids except that a larger sample of serum was necessary, it was not exposed to alkali, and the hexane extract was washed with dilute acid. For example, 1.5 ml of methanol, 5 ml of hexane and 0.5 ml of 1 *M* phosphoric acid were added with brief mixing after each addition to 0.4 ml of serum. The mixture was shaken vigorously for 1 min and centrifuged. The hexane phase was washed twice with 2 ml of 0.1 *M* phosphoric acid and then transferred to a 5-ml conical tube. The fatty acids were finally extracted from the hexane extract with 5 μl of TMTFTH.

Total saponifiable fatty acids from 5 μl of serum. This procedure was identical with that described above for analyzing 100-μl serum samples except for some miniaturization. The serum sample was digested with 0.2 ml of methanolic potassium hydroxide in a 5-ml glass-stoppered conical centrifuge tube, acidified with 0.3 ml of 1 *M* phosphoric acid, and extracted into 2 ml of hexane. The hexane phase was extracted with 5 μl of TMTFTH in a 3-ml glass-stoppered conical centrifuge tube (Pyrex No. 8061). The results are shown in Fig. 1.

Total saponifiable fatty acids from needle biopsy tissue specimens. The biopsy specimen (*e.g.*, 3–5 mm × 1.0–1.2 mm of liver) was placed in the bottom of a 5-ml glass-stoppered conical centrifuge tube. The sample was digested with 0.2 ml of the methanolic potassium hydroxide for 30 min at 65°. The contents of the tube were

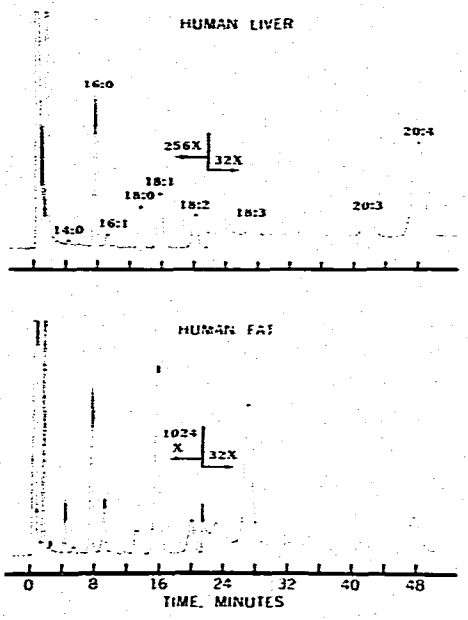

Fig. 2. Gas–liquid chromatography of fatty acid methyl esters derived from needle biopsy samples of human liver and adipose tissue. The samples were taken from *post-mortem* specimens by means of a Menghini liver biopsy needle, 1.20 mm I.D., and were processed as described in the text.

TABLE I

RECOVERIES OF FATTY ACIDS AFTER SAPONIFICATION AND METHYL ESTERIFICATION OF A REFERENCE ESTER MIXTURE

Applied Science Fat and Oil Reference No. 6 was used in these studies. Response factors obtained from ten analyses of a dilution of the reference mixture were applied to ten analyses of each of the esterification procedures.

Fatty acid	*Label data (%)*	*Found (% ± standard deviation) after saponification and esterification with*			
		BF_3	*HCl*	*H_2SO_4*	*TMTFTH*
14:0	2.0	1.97 ± 0.06	1.86 ± 0.11	2.36 ± 0.10	1.97 ± 0.07
16:0	30.0	30.01 ± 0.40	30.07 ± 0.50	29.61 ± 0.80	30.17 ± 0.36
16:1	3.0	3.06 ± 0.28	2.72 ± 0.06	3.33 ± 0.11	2.99 ± 0.06
18:0	14.0	13.99 ± 0.12	14.43 ± 0.20	10.87 ± 0.43	13.69 ± 0.08
18:1	41.0	41.20 ± 0.26	41.29 ± 0.38	42.61 ± 0.50	41.15 ± 0.36
18:2	7.0	6.92 ± 0.12	6.72 ± 0.13	7.87 ± 0.20	7.03 ± 0.10
18:3	3.0	2.98 ± 0.06	2.84 ± 0.08	3.67 ± 0.17	3.05 ± 0.06

mixed on a vortex mixer several times during the course of the digestion. The remainder of the procedure was identical with the 5-μl serum procedure. The results are shown in Fig. 2.

RESULTS

In order to determine the reliability of our procedure, two approaches were used. In the first approach, a mixture of fatty acid methyl esters of known qualitative and quantitative composition was chromatographed. The elution pattern of this sample was compared with the chromatograms obtained after saponification, extraction, and re-methylation by a number of established esterification procedures and by our new method. Table I shows the results of this study. It can be seen that our method yields essentially the same results as obtained with the original esters and is at least as good as other esterification procedures.

TABLE II

ANALYSIS OF FATTY ACIDS FROM POOLED HUMAN BLOOD SERUM

Fatty acid	*Normalized peak area ± standard deviation*	
	*Folch–NaOMe–BF_3**	*TMTFTH***
14:0	0.94 ± 0.04	0.92 ± 0.03
16:0	25.16 ± 0.23	25.31 ± 0.19
16:1	4.01 ± 0.03	4.02 ± 0.04
18:0	7.09 ± 0.09	6.70 ± 0.23
18:1	32.60 ± 0.23	32.21 ± 0.09
18:2	22.23 ± 0.22	22.62 ± 0.08
20:3	1.43 ± 0.08	1.46 ± 0.03
20:4	6.55 ± 0.20	6.75 ± 0.05

* Results from analyzing six 1.0-ml aliquots of serum.
** Results from analyzing six 100-μl aliquots of serum.

In the second approach, used to determine the reliability of our new procedure, a sample of pooled human blood serum was analyzed several times by an accepted conventional procedure —extraction according to Folch *et al.*[1] followed by sodium methoxide methanolysis[2] and boron trifluoride methylation[3]— and by the procedure described here for analyzing 100-μl serum samples. The results of this study are presented in Table II and show that our new method yields data that are in excellent agreement with a well established procedure. Fig. 1 shows the chromatograms obtained.

The determination of the relative amounts of the individual fatty acids in all these analyses were performed by multiplying the peak height of each ester by its retention time as measured from the point of injection. We find that the precision obtained by this means is twice as good as that obtained through the use of a disc integrator on the same chromatograms. This is in agreement with the findings of Grant and Clarke[4].

DISCUSSION

Methylation of carboxylic acids by thermal degradation of their quaternary ammonium salts in the injection port of a gas chromatograph was first demonstrated by Robb and Westbrook[5], who injected tetramethylammonium hydroxide solutions of a number of acids into a hot vaporizer and obtained chromatograms of the corresponding methyl esters. Downing and Greene[6] showed that the polyunsaturated fatty acids did not survive this treatment unless the excess of alkali was exactly neutralized with acetic acid. Brochmann-Hanssen and Oke[7] showed that trimethylphenylammonium hydroxide accomplished a more efficient N,N-dimethylation of barbiturates than did the tetramethylammonium reagent. They attributed this difference to the fact that dimethylaniline is a better leaving group than trimethylamine. Cakes and Willis[8] went one step further and used trimethyl(*α*,*α*,*α*-trifluoro-*m*-tolyl)ammonium hydroxide to methylate urinary carboxylic acids since dimethyl(trifluoro)toluidine should be a better leaving group than dimethylaniline. In support of this claim, they demonstrated that a lower injector temperature could be used with their reagent than was necessary with the trimethylphenyl reagent, which in turn was lower than the temperature necessary to produce good yields of methylated compounds from the tetramethylammonium reagent.

It appears that there is a considerable difference in the alkalinities of these three reagents. Tetramethylammonium hydroxide causes essentially complete destruction of the polyunsaturated fatty acids, trimethylphenylammonium hydroxide causes less destruction, and the trimethyl(trifluorotolyl)ammonium hydroxide causes even less. When a stoichiometric excess of methyl propionate is mixed with the alkaline samples, the recoveries of the polyunsaturated fatty acids improve. The recovery of the polyunsaturated fatty acids is not complete when methyl propionate is mixed with the two more alkaline quaternary ammonium hydroxide extracts, but when methyl propionate is mixed with the trimethyl(trifluorotolyl)ammonium hydroxide extract as described in our procedures, no degradation of linoleate, linolenate or arachidonate can be detected. Apparently the excess alkalinity of the reagent is neutralized as a consequence of the hydrolysis of the propionate ester in the vaporizer of the chromatographic unit. We find this "ester neutralization" to be much more convenient than trying to add exactly the right amount of acetic acid.

The lower vaporizer temperature that can be used with Oakes and Willis' reagent has the advantage that any gas chromatographic unit with a vaporizer that can be kept at 240° produces sufficient conversion of the quaternary salts into their esters for good quantitative data. The need to heat the vaporizer to 360°, as is the case with the tetramethylammonium salts, is not possible with some equipment.

The extraction technique used in our procedure, where the acidic and neutral organic fractions are extracted from the acidified sample into an organic solvent and then the acids only are extracted from the organic phase into a very small volume of the alkaline methylating agent, is patterned after the successful methods developed in this laboratory for the analysis of acidic drugs[9,10]. The use of a very small volume of the quaternary ammonium hydroxide yields a sufficiently concentrated sample to allow direct injection into the chromatographic unit without any time-consuming and destructive evaporation steps.

Although only limited studies have been performed with an internal standard, the results have been encouraging. It appears that when known concentrations of heptadecanoic acid are added to the alkaline hydrolysis reagent, the results obtained can be accurately expressed as the weight of each measured fatty acid per unit weight or volume of sample.

The rate at which the sample is expelled from the syringe into the vaporizer of the chromatographic unit has an influence on the width of the solvent response, the peak heights and the resolution of the peaks. This is true for pre-formed derivatives as well as for derivatives formed in the injector. In some situations, slow injection shows dramatic advantages over rapid injection[10]. In the current study, only slightly taller peaks were obtained on slow injection, so we used the conventional rapid injection technique in all our analyses. We strongly recommend that the best rate of injection be determined for a given instrument for each set of chromatographic conditions used, as the geometry of the inlet system, the injection block temperature, the back-pressure of the column, and the flow-rate of the carrier gas all appear to influence the elution pattern.

TABLE III

COMPARISON OF FATTY ACID ANALYSES OF 100-μl AND 5-μl SERUM SAMPLES

Fatty acid	*Normalized peak area ± standard deviation*		
	*100 µl serum**	*5 µl serum**	
		Uncorrected	*Corrected***
14:0	0.96 ± 0.03	1.48 ± 0.13	0.85 ± 0.14
16:0	25.64 ± 0.14	27.55 ± 0.87	26.43 ± 0.97
16:1	3.87 ± 0.06	4.07 ± 0.09	3.11 ± 0.14
18:0	6.87 ± 0.21	8.40 ± 0.48	7.83 ± 0.51
18:1	32.64 ± 0.08	31.71 ± 0.52	32.16 ± 0.55
18:2	22.12 ± 0.10	20.11 ± 0.67	22.07 ± 0.77
18:3	0.78 ± 0.04	0.73 ± 0.10	0.83 ± 0.11
20:3	1.35 ± 0.04	1.21 ± 0.10	1.37 ± 0.13
20:4	5.83 ± 0.06	4.75 ± 0.15	5.36 ± 0.16

* Results from analyzing six aliquots of serum.

** Obtained by subtracting the mean peak areas arising from the procedure performed on six 5-μl aliquots of water.

We have described two procedures for analyzing different-sized samples of blood serum (100 and 5 μl). The analyses obtained by means of the 5-μl procedure vary only slightly from those obtained with the 100-μl procedure, as can be seen in Table III. Some improvement is obtained when corrections are made by subtracting the small peaks arising from reagent blanks. These corrected data are also found in Table III, and it can be seen that useful data can be obtained in this way. The limiting factors influencing the accuracy and precision in these ultramicro analyses are "ghosting" or "memory" effects and any trace impurities in the reagents. The former problem is most serious when the analyses are performed on dilute specimens immediately after concentrated samples. It appears that satisfactory corrections can be made through the use of blank samples.

The method described in this paper was designed for the determination of the total saponifiable fatty acids (fatty acid esters and free fatty acids) in small biological specimens. Neither this procedure nor the double esterification of the isolated lipids of the control procedure yields methyl esters from the fatty acid amides of the sphingolipids. Such amides require prolonged heating in acid for release.

Cholesteryl esters, phospholipid esters, triglyceride esters and free fatty acids are all quantitatively converted into their methyl esters by our new procedure when the saponification step in included.

Any plasmalogens in the specimens do not yield dimethylacetals by our procedure. Although the enol ethers could be cleaved during the acidification of the saponified sample, and the released aldehydes could be extracted into the hexane phase, they would not be extracted into the aqueous TMTFTH phase.

Extracted lipids and food fats can also be analyzed by our new procedure if sufficiently small samples are taken. Obviously it is important that sufficient TMTFTH be used for extraction of the fatty acids. Insufficient TMTFTH would result in loss of some of the fatty acids and might even result in the deposition of free fatty acids in the chromatographic column where they would contribute "memory" or "ghost" peaks.

REFERENCES

1 J. Folch, M. Lees and G. H. Sloane Stanley, *J. Biol. Chem.*, 226 (1957) 497.
2 F. E. Luddy, R. A. Barford and R. W. Riemenschneider, *J. Amer. Oil Chem. Soc.*, 37 (1960) 447.
3 L. D. Metcalfe, A. A. Schmitz and J. R. Pelka, *Anal. Chem.*, 38 (1966) 514.
4 D. W. Grant and A. Clarke, *Anal. Chem.*, 43 (1971) 1951.
5 E. W. Robb and J. J. Westbrook, III, *Anal. Chem.*, 35 (1963) 1644.
6 D. T. Downing and R. S. Greene, *Anal. Chem.*, 40 (1968) 827.
7 E. Brochmann-Hanssen and T. O. Oke, *J. Pharm. Sci.*, 58 (1969) 370.
8 T. R. Oakes and C. E. Willis, *Amer. Ass. Clin. Chem. 24th National Meeting, August 20–25, 1972*, Abstr. 023, p. 25.
9 J. MacGee, *Anal. Chem.*, 42 (1970) 421.
10 J. MacGee, *Clin. Chem.*, 17 (1971) 587.