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Note

Methylation of skin surface lipid free fatty acids

Comparison of methods

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Free fatty acids (FFA) may be important in the development of acne[1] and we are therefore investigating skin surface FFA composition in patients with this condition. FFA are usually converted to methyl esters before analysis by gas chromatography (GC). It has been stated[2] that methylation ideally should be simple, rapid and quantitative, and should give rise to no structural changes or side products.

To this end a variety of reagents have been employed including methanol with acid catalyst[3,4], methanol with boron trifluoride catalyst[5], and diazomethane[6] under experimental conditions ranging from high-temperature-and-pressure vapour-phase methylation[7] to room-temperature methylation using ion-exchange resin[6]. On-column conversion to methyl esters of the tetramethylammonium salts of fatty acids has also been employed in GC[8]. However, few authors have compared the completeness of methylation effected by different methods on complex mixtures of FFA. It is possible that completeness of methylation in a FFA mixture may vary with the composition of the mixture. Since there are no such data on completion of methylation using surface lipid fatty acids, we thought that this should be investigated. We therefore compared four methods of methylation, the methods being chosen for experimental convenience. Methods employing diazomethane were excluded from this study because of the dangerous nature[9] of this reagent, which, we consider, makes it undesirable for routine use in a hospital environment.

EXPERIMENTAL AND RESULTS

The FFA were derived from surface lipid, this being collected either by absorbent papers or by diethyl ether-soaked sponges. The total lipids were then subjected to Florisil column chromatography[10] to separate the FFA. A stock solution was made by dissolving 30 mg of FFA in 100 ml of chloroform.

The four methylation methods we used were: (1) 1% H_2SO_4/methanol methylation; (2) 5% HCl/methanol methylation; (3) 14% BF_3/methanol methylation; (4) H_2SO_4–chloroform–methanol (1:100:100) at 160°. All solvents and the methylation reagents were of AnalaR grade and were dehydrated before use by overnight standing in the presence of anhydrous sodium sulphate.

TABLE I

METHYLATION METHODS USED

Method No.	*Methylating reagent*	*Methylating conditions*	*Method modified from ref.*
1	1 ml benzene, 4 ml 1% H_2SO_4 in methanol	Incubate in water-bath at 60° for 4 h	4
2	1 ml benzene, 4 ml 5% HCl in methanol	Incubate in water-bath at 60° for 4 h	3
3	0.5 ml 14% BF_3 in methanol	Incubate in water-bath at 60° for 2 h	5
4	0.75 ml H_2SO_4–chloroform–methanol (1:100:100)	Incubate in oven at 160° for 30 min in aluminium cylinder	7

In methods 1, 2 and 3, 2.6-ml aliquots of stock FFA solution were pipetted into 15-ml test tubes with ground glass stoppers and the solution evaporated to dryness under slow nitrogen streams to leave a residue of 800 μg of FFA per tube. In method 4, 800 μg FFA were similarly added to a 0.5-ml hard glass tube. The dry FFA residues were methylated as summarised in Table I. The aluminium cylinder used as reaction tube container in method 4 is represented in Fig. 1.

After methylation, whatever method was used, the lipids were extracted from the reaction mixtures with 5 ml of AnalaR petroleum ether (b.p. 40–60°). The extracts were gently evaporated to dryness under slow nitrogen streams and the lipids taken up in 0.02 ml of chloroform.

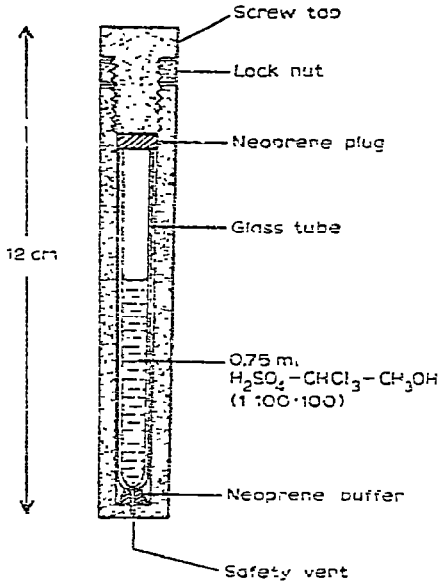
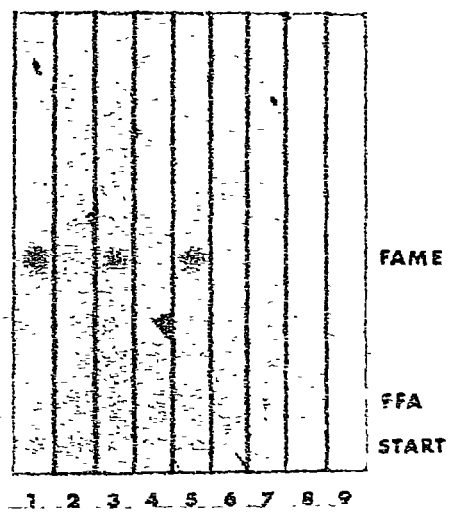


Fig. 1. Aluminium cylinder containing hard glass reaction vessel set-up for high-temperature-and-pressure vapour-phase methylation.

To assess completeness of methylation it was necessary to measure how much unmethylated FFA remained. This was done by thin-layer chromatography (TLC). Five-microlitre loads of chloroform solution were spotted onto the baseline of 20 cm × 20 cm Kieselgel 60 TLC plates (Merck) scored to give tracks 14 mm wide. Standard loads of FFA were added for calibration. Each plate was run in hexane–diethyl ether–acetic acid (80:20:2) to a solvent-front-above-baseline height of 10 cm and then in hexane–diethyl ether (95:5) to a height of 18 cm.

The plates were then developed by a brief dip in 10% sulphuric acid followed by heating at 180° for 15 min. Fatty acids and methyl esters were identified by reference to authentic samples. The intensities of spots representing unconverted FFA and standard loads of FFA were measured using a Joyce-Loebl Chromoscan.

Figs. 2 and 3 show TLC assessment of completeness of methylation for the four methods. The calibration plots for known FFA loads *vs.* Chromoscan counts, represented in Fig. 4 and Table II, were calculated by the method of least squares and the regression coefficients ($r = 0.985$ for plate A; $r = 0.990$ for plate B) indicate satisfactory fit of the points to straight lines.



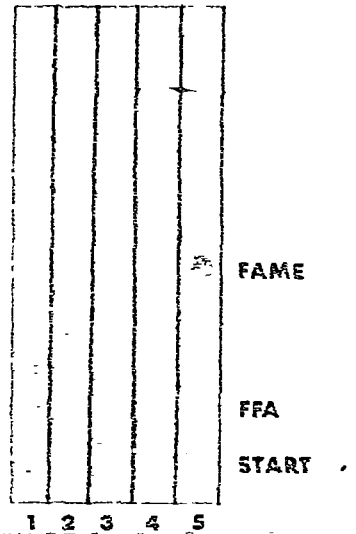


Fig. 2. TLC analysis of the results of methylation methods 1, 2, and 3. Lane 1: method 1; lane 2: blank (no FFA) for method 1; lane 3: method 2; lane 4: blank for method 2; lane 5: method 3; lane 6: blank for method 3; lanes 7, 8, 9: calibration loads of 10, 5, and 2.5 μg, respectively, of FFA.

Fig. 3. TLC analysis of the results of methylation method 4. Lane 1: blank (no FFA); lanes 2, 3 and 4: calibration loads of 10, 5, and 2.5 μg, respectively, of FFA; lane 5: method 4.

It will be noted that there are two calibration plots, one for methods 1–3 (TLC plate A) and the other for method 4 (TLC plate B). This is because each plate must have its own set of standards and a calibration plot as the degree of charring produced by the sulphuric acid and heating varies slightly between plates.

Table III shows the Chromoscan counts for quantities of FFA left unmethylated and the weights of FFA (read from the calibration plots) which these represent. All methods gave a very high conversion rate (all >97%) of FFA to methyl esters.

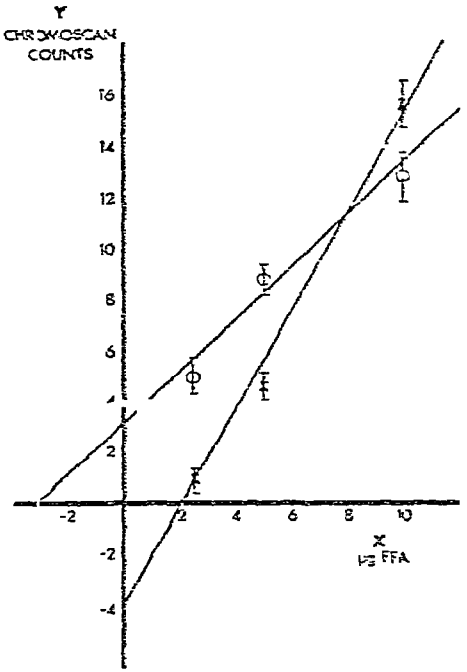


Fig. 4. Calibration plots for TLC plates A (○) and B (×) of known FFA loads *vs.* Chromoscan counts.

TABLE II

CHROMOSCAN READINGS FOR STANDARD LOADS OF FFA

Plate A		*Plate B*	
Chromoscan counts (mean ± S.E.M. for 5 readings)	*FFA load (µg)*	*Chromoscan counts (mean ± S.E.M. for 5 readings)*	*FFA load (µg)*
12.8 ± 0.98	10	15.6 ± 1.15	10
8.8 ± 0.95	5	4.6 ± 0.46	5
5.0 ± 0.63	2.5	1.8 ± 0.33	2.5

TABLE III

CONVERSION OF FFA TO METHYL ESTERS

No.	*Method*	*Initial FFA load (µg)*	*Residual FFA Chromoscan counts (mean ± S.E.M. for 5 readings)*	*FFA (µg) from graph*	*% methylation (R/L × 100%)*
1	1% H_2SO_4/methanol	200	6.8 ± 0.82	3.5	97
2	5% HCl/methanol	200	3.2 ± 0.33	0.2	99
3	14% BF_3/methanol	200	2.2 ± 0.33	−0.7	99
4	Cylinder	200	6.6 ± 0.22	5.4	97

Analysis of values of Chromoscan counts for residual FFA by the t test showed that there was no significant difference between the results given by 1% H_2SO_4 methylation and high-pressure methylation ($t = 0.2795$; $2P > 0.80$) or between 5% HCl methylation and 14% BF_3 methylation ($t = 2.5$; $2P > 0.05$). However, if the results of 1% H_2SO_4 methylation and high-pressure methylation are combined and compared with the results of 5% HCl and 14% BF_3 methylation, then significance was achieved ($t = 11.54$; $2P < 0.001$).

DISCUSSION

The four methods we investigated are commonly used methods of methylation and all gave satisfactory complete methylation of sebum fatty acids. Boron trifluoride methanolysis and HCl methanolysis gave the best results. Because of the shorter experimental time taken by the former method (2 h compared with 4 h) it was concluded that the boron trifluoride method was preferable.

Comparison of the results obtained with those of other authors is difficult since the methods used for assessment of percentage methylation differ from study to study. However, it is worth noting that boron trifluoride methanolysis has been shown[5] to give high values of percentage methylation (97% for caproic acid, 99% for caprylic acid, and 99% for capric acid) similar to those obtained in this study (>97%). The comparative study by Vorbeck *et al.*[6] of the efficacy of diazomethane methanolysis, HCl-catalysed methanolysis and boron trifluoride methanolysis as methylating methods also gave results in the 90's for percentage methylation of prepared mixtures of fatty acids, containing up to five fatty acids of chain lengths C_{10} to C_{18}. However, these results were obtained using substrates much simpler than the FFA fraction of sebum, which could contain more than fifty individual fatty acids[11], and this difference might be a further reason to avoid direct comparisons.

The validity of our results depends upon the reliability of the Chromoscan readings of the FFA spots. Therefore, failure of the two calibration plots to intersect the origin requires explanation. Fig. 4 shows that the standard errors of the mean of sets of five Chromoscan readings of the spots given by FFA loads in the range of 2–10 μg are not prohibitively large. Further, the correlation coefficients of the regression lines calculated indicate satisfactory fit of the points of the lines.

Thus it may be concluded that used in this way the Chromoscan instrument gives usable readings of spot intensity for FFA loads in this range and that spot intensity is a valid measure of the relative size of different FFA loads. The reason for non-intersection of the origin in the calibration plots probably resides in the charring method, such that plates are either incubated too long when no FFA zones are darkened or plates are incubated too briefly when incomplete charring of FFA takes place. The results would be to push all points up or down, respectively, on the y axis (Fig. 4), forcing the regression line to miss the origin.

The dehydration of reagents before methylation is of great importance. Preliminary work in this department has shown that failure to dehydrate reagents with anhydrous sodium sulphate, as described earlier, leads to a considerable decrease in percentage methylation. These findings are in agreement with the suggestion of Stoffel *et al.*[3] that the nucleophilic attack of methanolic oxygen on the carboxylic acid carbon of fatty acid is competitively inhibited by the more electro-negative oxygen of water.

We decided that the highly satisfactory results given by the methods tested justified our original decision not to use diazomethane. The boron trifluoride method described in the study is now in regular use in our department.

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