notes on methodology

Analysis of fatty acids of human red cells without lipid extraction

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SUMMARY Exposure of human red cells to 2 N HCl for 18–20 hr at 110°C appears to release the total fatty acid, which can then be esterified for GLC analysis. This technique is simpler and may be more reliable than the conventional methods that depend on lipid extraction of the red cells.

KEY WORDS fatty acids · human red cells · hydrolysis · sphingomyelin · gas–liquid chromatography

THE USEFULNESS OF fatty acid analysis of red cells for large-scale nutritional studies in man has been suggested (1). One of the difficulties in such an analysis has been the quantitative extraction of the red cell lipid prior to the synthesis of the fatty acid methyl esters. Apparently at least three such extractions are required to remove over 95% of the phospholipid from human red cells (2); the procedure is time-consuming and tedious, and the degree of extraction may be variable. The repeated manipulation of the red cell residue, furthermore, increases the possibility of lipid peroxidation. In the present study, we found that the red cell fatty acid can be isolated by a relatively simple procedure without prior lipid extraction. The fatty acid can then be esterified for GLC analysis which provides a rapid and reliable method, useful for screening, for determining the total fatty acid composition of human red cells.

Methods. Venous blood samples from 10 adult subjects who were apparently healthy (except for schizophrenia in two of them) were analyzed; the individual samples were anticoagulated with EDTA and the red cells were washed three times with saline buffered to pH 7.4, as described previously (3).

A 0.5 ml sample of red cells was pipetted into a mixture of 5 ml of 2 N HCl (aqueous) and 0.2 ml of methanol [the methanol containing 0.2 g of 2,6-di-*tert*-butyl-*p*-cresol (BHT, Ionol CP, Shell Chemical Corp, New York) per 100 ml] in an 18 × 150 mm Pyrex test tube. The mixture was shaken and bubbled out with "prepurified" nitrogen (Ohio Chemical Company, New York). The tube was sealed with a flame and put in an oven at 110°C for 18–20 hr. After removal from the oven, the tube was allowed to cool, the top was cracked off, and the mixture was extracted twice with 2 ml and twice with 1 ml of pentane containing 5 mg of BHT per 100 ml. Vigorous shaking of the mixture with the pentane was required for adequate extraction.

The pentane was put into a 10 ml ampoule and taken to dryness with a nitrogen jet, and 1 ml of boron trifluoride–methanol reagent (Applied Science Laboratories, Inc., State College, Pa.) was added for the production of methyl esters by the method of Morrison and Smith (4), as previously described (3). The heating step in this procedure was carried out for 30 min. In order to separate the methyl esters from substances which might interfere with their analysis by GLC, we chromatographed the methyl ester mixture on a 0.5 mm layer of Silica Gel HR (Brinkmann Instruments, Inc., Westbury, N.Y.) with toluene containing 50 mg of BHT per 100 ml. The methyl ester spot was scraped into a 12 ml conical centrifuge tube and eluted once with 2 ml and twice with 1 ml of chloroform containing 5 mg of BHT per 100 ml. The eluate was dried with a nitrogen jet and the residue was dissolved in hexane for GLC, which was performed as previously described (3).

Another sample of the washed red cells of each subject was extracted three times with methanol–chloroform 1:1 as previously described (3). This lipid extract was subsequently handled in exactly the same manner as the corresponding hydrolysate extract. The dimethyl acetal spot was not separated discretely on the thin-layer plate by either method and was not analyzed.

Results and Discussion. The mean values and standard deviations for all of the fatty acids comprising > 1% of the total by the hydrolysis and extraction methods are shown in Table 1. The close agreement between the results obtained by the two methods validates the hydrolysis technique as a reliable method for the estimation of the total fatty acids of human red cells. The greater simplicity and reduced manipulation required in this procedure, moreover, make it useful as a screening technique.

Although the differences in values for individual fatty acids between the two methods were usually slight, for at least five of the fatty acids listed (Table 1), i.e., 16:0, 18:1ω9, 18:2ω6, 22:0 + 20:3ω6, and 24:0, they were

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Abbreviations: BHT, 2,6-di-*tert*-butyl-*p*-cresol; GLC, gas–liquid chromatography. Fatty acids are designated by number of carbon atoms:number of double bonds, and the digit after the ω states the number of carbon atoms from the methyl end of the acyl chain to the middle of the nearest double bond.

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TABLE 1 COMPARISON OF VALUES* FOR MAJOR RED CELL FATTY ACIDS BY TWO METHODS

Fatty Acid	Method		P†
	Extraction	Hydrolysis	
	g/100 g of fatty acids		
16:0	19.2 ± 0.8	19.9 ± 0.9	<0.01
18:0	15.7 ± 1.0	15.6 ± 0.8	
18:1ω9	13.9 ± 1.1	12.9 ± 0.9	<0.001
18:2ω6	9.6 ± 0.9	9.0 ± 0.7	<0.01
22:0 + 20:3ω6	3.1 ± 0.5	3.6 ± 0.4	<0.001
20:4ω6 + 22:1ω9	15.4 ± 1.0	14.9 ± 0.4	
24:0	4.2 ± 0.8	5.4 ± 0.4	<0.001
22:4ω6 + 24:1ω9	7.6 ± 1.0	7.9 ± 0.8	
22:5ω3 + 24:2ω6	2.5 ± 0.3	2.4 ± 0.5	
22:6ω3 + 26:0	5.0 ± 0.7	4.6 ± 0.7	

* Values are shown as mean ± SD of results from 10 "normal" subjects.

† Probability that the value for both methods is the same; average difference between the methods on paired samples was tested for significance by "*t*" test (9 degrees of freedom).

statistically significant. Of these, the values for 16:0, 22:0 + 20:3ω6, and 24:0 were higher by the hydrolysis method. Since 24:0, which showed the largest difference, is found almost exclusively in the sphingomyelin of human red cell phospholipid (3), this difference suggests that sphingomyelin or a nonphospholipid source of 24:0, such as glycosyl ceramide, may not have been extracted completely by the extraction method. In support of this possibility were (*a*) the suggestively higher values by the hydrolysis method for 20:0, 22:0, and 24:2, which are also found almost entirely in sphingomyelin (3) [16:0, about 30% of which in the total red cell phospholipid is in sphingomyelin (3), was also higher by the hydrolysis method]; and (*b*) the considerably higher standard deviation for 24:0, as well as for most of the other fatty acids, by the extraction method, which suggests a variable degree of extraction. An alternative explanation is that the values for the sphingomyelin fatty acids, which are largely saturated (3), are relatively higher by the hydrolysis method because of destruction of polyunsaturated fatty acids. However, the values for major polyunsaturated fatty acids such as 20:4ω6 and 22:6ω3, which are present in minor quantities if at all in sphingomyelin (3), were not statistically significantly lower in the hydrolyzed samples. Furthermore, another major saturated fatty acid, 18:0, which makes up only about 5–6% of the sphingomyelin fatty acid (3), had essentially the same value by both methods. The two fatty acids that were significantly lower by the hydrolysis method, 18:1ω9 and 18:2ω6, which are found predominantly in the red cell lecithin (3) and each constitute less than 1% of sphingomyelin fatty acids, may have been lower at least partly because of dilution. These results suggest that the extraction of sphingomyelin or of a nonphospholipid source of fatty acid may be variable by conventional methods, and that the hydrolysis method for fatty acid analysis is not only much easier to carry out but may also be more reliable and reproducible.

Aliquots of two of the red cell samples were also hydrolyzed for 7 hr; the results were the same as with the 20 hr hydrolysis, except that the values for 20:4ω6 and 22:6ω3 were slightly higher (24:0 showed no difference). In view of the suggestively lower values for 20:4ω6 and 22:6ω3 by the hydrolysis method shown in Table 1, the possibility arises that slight destruction of polyunsaturated fatty acids might have occurred during the prolonged hydrolysis and that 7 hr of hydrolysis may be preferable.

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