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Titration of free fatty acids of plasma: a study of current methods and a new modification*

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SUMMARY

Dole's titrimetric method for determination of the free fatty acids of plasma has been modified to improve its specificity. The fatty acid solution which is to be titrated is first washed with 0.05 per cent H_2SO_4 and thus freed of lactic acid and of an acetone-insoluble material which interferes. With this change, the Dole procedure yields results which agree well with those of Gordon's method.

Two titrimetric methods for determination of free fatty acids (FFA) of plasma, those of Dole (1) and Gordon (2), are now in wide use. Unlike the latter, the Dole method requires only moderate amounts of time, skill, and special apparatus, but it is not strictly specific for fatty acids (3). Specifically, plasma lactate interferes (2, 3), particularly at the high levels obtained with acute exercise.

A procedure was devised to wash out interfering substances and to improve the specificity of the Dole method. In testing the modified procedure, efforts were made to account for the change in apparent FFA values observed with washing.

METHODS

The Modified Procedure. It is convenient (but not essential) to use twice the volumes of plasma and of reagents specified in the Dole procedure. Thus to 2 ml. heparinized plasma in a glass-stoppered test tube are added, with shaking, 10 ml. "extraction" mixture (40 parts by volume of isopropanol, 10 parts heptane, and 1 part 1 N H_2SO_4); then 6 ml. heptane and 4 ml. water are introduced, and the mixture is shaken for at least 2 minutes. A 4- or 5-ml. aliquot of the upper, or "heptane," layer is removed to a glass-stoppered centrifuge tube and there vigorously shaken for 5 minutes with an equal volume of 0.05 per cent aqueous H_2SO_4. The tube is then centrifuged at about 300 × *g* for 5 minutes. To a final tube containing 1 ml. Dole's "titration" mixture is transferred 3.0 ml. of the washed heptane layer, and it is then titrated with 0.018 N NaOH while being agitated with a stream of nitrogen. The heptane layers from appropriate titration blanks and palmitic acid standards are similarly washed before titration.

Other Methods. Since lactic acid and perhaps phospholipids interfere in the Dole procedure, the concentrations of these substances were determined in washed and unwashed aliquots of heptane layer from plasma extract.

In the lactate analyses a measured volume of the heptane layer was evaporated under a stream of air at room temperature. The residue was shaken up into a water suspension, which was treated with $Ba(OH)_2$ and $ZnSO_4$ (4) and filtered. The filtrate was analyzed for lactate by the method of Barker and Summerson (5).

In the lipid phosphorus analyses the residue from the heptane layer was almost completely dissolved in alcohol-ether 3:1, and the solution filtered. Aliquots were dried and digested with hot H_2SO_4 and H_2O_2 (6). Final color development was by the method of Fiske and Subbarow (7).

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A method was devised and tested for the evaporation of lactic acid from the heptane layer of plasma extract. The heptane layer was evaporated to near dryness, with continuous shaking, under the reduced pressure of a water aspirator. With the vacuum attachment still on, tubes were then warmed by movement in a water bath (approx. 60°C) until the last traces of solvent disappeared. The residue was dissolved in 2.0 ml. isopropanol, which was then evaporated in the same manner, after which the isopropanol treatment was repeated once.

The above procedure was tested in the following manner for its efficiency in removing lactate: Samples of the same plasmas were extracted by the Dole method with and without the addition of lactate; the heptane layers were subjected to the low-pressure treatment, reconstituted with heptane, and titrated. Titration data revealed the loss of about 90 per cent of the lactic acid originally present in the heptane layer.

Portions of lactate-depleted residue from the heptane layer of plasma extract were also largely freed of phospholipid by acetone precipitation (8). Specifically, the residue from the heptane extract from 4.0 ml. plasma was dissolved in 0.5 ml. petroleum ether (b.p. 30°-60°C); and 4.2 ml. acetone was added with shaking. Phospholipid precipitated during storage overnight with dry ice. The tube was then centrifuged, with small quantities of dry ice added to the tube carrier. One 3-ml. aliquot was titrated directly; a second portion after being washed with an equal volume of 0.05 per cent aqueous H_2SO_4.

In titrating the heptane layer residue, the solute was made up to only one-quarter to one-half the original volume, so that the sensitivity of titration was increased. In preparing titration blanks, similar volumes of heptane layer of water extracts were treated in a manner identical to those from plasma extract.

In procedures with palmitic acid-1-C^{14} the samples were added to metal planchets, evaporated to dryness under an air stream, and counted with a thin-window Geiger-Müller tube.

RESULTS

The original Dole method for FFA yields higher values than the elaborate Gordon procedure. The difference in some cases amounts to 100 to 200 μmoles per l. or up to 50 per cent. The modified Dole method, as described above, and the Gordon procedure were applied to the same 10 plasma samples. As indicated by Table 1, results by the two methods showed no significant difference.

TABLE 1. COMPARISON OF METHODS

Plasma Sample	Modified Dole Procedure	Gordon Method	Difference
	μmole/l.	*μmole/l.*	*μmole/l.*
1	271	298	−27
2	447	485	−38
3	678	731	−53
4	264	267	−3
5	258	219	+39
6	196	175	+21
7	340	359	−19
8	720	682	+38
9	1369	1373	−4
10	441	270	+171
Mean difference ± standard error			+12.5 ± 20.1

Effects of Washing Dole's Heptane Layer. It was necessary to determine whether, in washing the heptane layer, the loss of long-chain fatty acids contributed significantly to the reduction in apparent fatty acid value. Palmitic acid-1-C^{14} was used as a prototype of long-chain fatty acids and, as the sodium soap, was shaken into solution with plasma. When the labeled plasma was extracted and the heptane layer washed in the standard way, 4 per cent of the label was found in the wash solution. It was, however, also noted that heptane persisted, despite centrifugation, emulsified in the acidified water.

It was reasoned that if the radiopalmitate of wash solution existed as minute droplets of the same heptane solution which constituted the upper phase and which was to be titrated, no error would be introduced in FFA estimation. By alternate freezing in acetone and dry ice and thawing at room temperature, the emulsion was broken. After centrifugation the previously entrapped heptane was found to have the same radioisotope concentration as that removed earlier, and the wash solution retained only 0.3 per cent of the total label. It is concluded that the small loss of long-chain fatty acids in the wash procedure introduces no appreciable error.

Interference by lactic acid was also studied in the following manner: A titration blank was first prepared with a known amount of racemic lactic acid (from crystallized lithium lactate). The heptane layer was titrated and found to contain 1.5 per cent of that

acid before washing and essentially none afterward. Direct analyses of lactic acid were then made on two plasma samples and on the heptane layer obtained from the same plasmas by the original and modified Dole procedures. The data for these plasmas, as summarized in Table 2, indicate that the unwashed heptane layer of plasma extract contained at least 1.5 per cent of the lactate present and that one washing reduced this contamination by at least 80 per cent.

TABLE 2. LACTIC ACID IN PLASMA AND HEPTANE LAYERS *

	Plasma	Dole's Heptane	Washed Heptane
Subject A	1.98	0.044	.0079
Subject B	1.38	0.026	.0042

* Values are given as μmole per ml. of original plasma.

Each mg. of lactic acid per 100 ml. plasma contributed 1.5 to 2.0 μmoles per l. to the Dole FFA estimate. However, for most plasmas from resting subjects, removal of this amount of lactic acid from the heptane layer accounted for less than half of the change in titration after washing. Since it was noted that the heptane layer contained 25 to 30 per cent of the lipid phosphorus of plasma but lost nearly nine-tenths of that material with washing, it was suspected that phospholipid might interfere in the unmodified Dole method.

The total lipids (9) from two plasma samples were chromatographed on silicic acid columns, essentially by the method of Hirsch and Ahrens (10), and the phospholipid fraction was evaporated to dryness and extracted by the Dole solvent system. The heptane layer showed titratable acidity which was markedly reduced by the recommended wash procedure.

A second approach to the effect of phospholipids on the apparent FFA value was to determine the change in titration value when the heptane layer of plasma extract was freed, successively, of lactic acid and of phospholipid. The heptane layer was so treated to volatilize the lactic acid (and some fatty acid). Aliquots of the resulting residue were dissolved in heptane and titrated; other aliquots were first treated to remove phospholipid, then dissolved in heptane and titrated. Results in Table 3 show that removal of phospholipids reduces titratable acidity. In fact, after removal of both lactic acid and phospholipids, the residue yielded titration values approximating those obtained with the washed FFA method. Titration changes with washing were also pertinent; this change was greatest for the untreated heptane layer of plasma extract, less after evaporation of lactic acid, and minimal after the additional removal of phospholipids.

TABLE 3. TITRATABLE ACIDITY OF HEPTANE LAYER AFTER PARTIAL FRACTIONATION *

	Sample	Dole Procedure	After Washing
Untreated	1	.283	.235
	2	.258	.212
Vacuum-treated (to remove lactic acid)	1	.250	.229
	2	.243	.206
Vacuum-treated and freed of acetone-insoluble material	1	.239	.233
	2	.210	.200

* Values are given as μmole per ml. of original plasma.

It is concluded that an acetone-insoluble material, probably phospholipid, is extracted and titrated in the Dole method but is largely washed out in the modified procedure.

Recovery of Added Fatty Acids. The chief components of plasma FFA, sodium palmitate, oleate, and stearate (11), were dissolved in plasma, and their recovery by the modified Dole procedure was determined. Specifically, 1.2 μmoles of each soap was added to one of three 2.0-ml. aliquots of plasma; these and a fourth unsupplemented portion were analyzed for FFA. This set of four analyses was carried out on each of ten plasmas.

Results, as shown in Table 4, indicate that recoveries of each of the three fatty acids studied did not differ significantly and that the mean recoveries exceeded 90 per cent.

TABLE 4. RECOVERIES OF FATTY ACIDS ADDED TO PLASMAS

Fatty Acid	Number of Trials	Recoveries *
		per cent
Palmitate	10	93.6 ± 0.69
Oleate	10	93.5 ± 0.85
Stearate	10	92.8 ± 0.94

* Mean ± standard error.

Reproducibility of the Modified Dole Method. Reproducibility of the original and modified Dole procedures was determined and compared in terms of standard deviation of repeated analyses. A total of 38 analyses was carried out by each method on the same 12 plasma samples. By Dole's original method, a mean FFA of 689 μmoles per l. with a standard deviation of 11.9 was found; the modified method gave 584 $\pm$ 11.2 μmoles per l.

DISCUSSION

The modified Dole procedure, as described above, preserves most of the ease and reproducibility of the original Dole FFA method but greatly improves its specificity for fatty acids. In the original Dole procedure an acetone-insoluble lipid, probably a phospholipid, interferes significantly. Lactic acid of normal fasting plasma also brings about an error of several per cent, and this error increases as the lactic acid rises with exercise, psychological stress, diabetes, or infusion of epinephrine or norepinephrine. The recommended wash treatment minimizes interference by these two materials.

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