Automatic titration of free fatty acids

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SUMMARY FFA have been titrated automatically in a single-phase system, obtained by adding isopropanol to non-polar extracts. Good reproducibility and linearity were obtained in the titration of standard palmitic acid. Titration results for serum extracts were comparable with those obtained manually by an experienced worker.

Free fatty acids (FFA) in plasma or tissue are usually determined by extraction, distribution into an organic solvent, and titration with aqueous NaOH (1). Titration is performed in a two-phase system which contains FFA

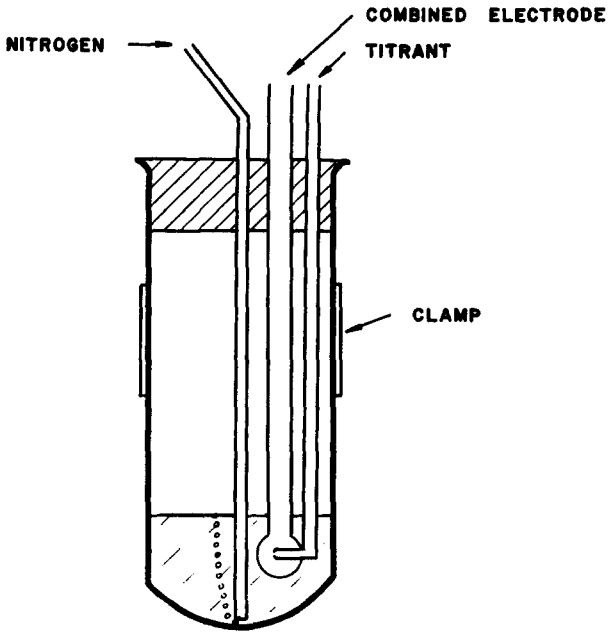

FIG. 1. Titrating assembly.

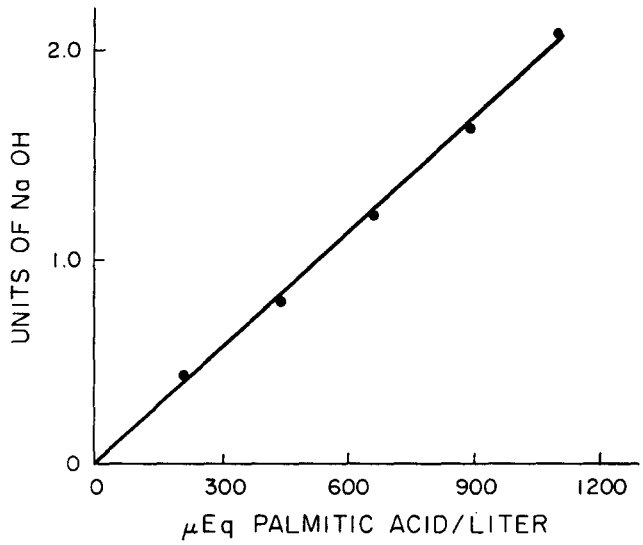

FIG. 2. Titration of standard palmitic acid. Known amounts of standard palmitic acid were processed according to the Dole extraction technique and titrated automatically. The units of NaOH on the ordinate refer to the amount of 0.015 N NaOH added as determined by a micrometer scale on the syringe burette.

in a nonpolar solvent and thymol blue indicator in a more polar ethanolic solution.

Under these conditions the titration is tedious and exacting because the end point, approached indirectly, is not distinct. Since the technique is not easily mastered, and even then remains somewhat individualized, it seemed desirable to convert the manual titration to an automatic one.

MATERIALS AND METHODS

Samples to be analyzed were extracted with a mixture of isopropanol, heptane, and 1 N H_2SO_4 (40:10:1) according to the method of Dole (1, 2). Heptane and water were added (1, 2) and automatic titration of the FFA in the upper organic layer was carried out by means of a Radiometer TTTlc titrator, 0.5 ml SBUla syringe burette, SBR2c titrigraph, and combined electrode, GK 2021c (all available from the London Co., Westlake, Ohio). Motor B of the titrigraph was set at 0.5 rpm and motor C at 4 rpm.

Figure 1 illustrates the positioning of the electrode relative to the glass delivery tubes for nitrogen and titrant. The titrant was delivered by the syringe burette through a delivery tip with an outlet barely able to admit a wire 0.127 mm in diameter which was 1–2 mm from the glass membrane of the combined electrode. Nitrogen was bubbled through an alcoholic NaOH scrubbing solution (1, 2) and then into the titrating vessel, where it prevented CO_2 absorption and mixed the titrant with the sample. Since the titrant had a tendency to settle, it was essential for adequate mixing to position the nitrogen delivery tip at the bottom of the tube.

The titrating vessel (Fig. 1), a 50-ml polypropylene tube, contained 3 ml of extract (1, 2), 1 ml of thymol blue indicator (1, 2), and 3 ml of isopropanol to produce a single-phase system.

Prior to titration the electrode was soaked in a blank solution for about 1 hr. To begin a titration the electrode and delivery tips were positioned in the sample, and after allowing sufficient time (40–60 sec) for the pH reading to become stable (at about 8.5), the titrator was turned on. Blanks and samples with less than 300 μEq of fatty acid per liter were titrated at a proportional band of 5; the majority of samples were titrated at a proportional band of 1. The proportional band refers to the number of pH units from the end point at which the titrator begins to reduce the delivery rate of titrant.

The titrant, 0.015 N NaOH, was delivered to a pH reading of 9.8, the approximate point at which thymol blue begins to turn green in this medium. The titrator was adjusted to turn off when no titrant had been added for 5 seconds.

The automatic method of titration was evaluated as follows. Samples of palmitic acid ranging from 0 to 1112 μEq/liter were processed according to the Dole extraction technique and titrated. In addition, standards containing 445 and 890 μEq/liter were titrated in duplicate 25 times. One-milliliter aliquots of sera from nine healthy individuals were also extracted and titrated automatically. At a later date the sera were extracted and titrated manually by an individual who has performed these titrations routinely for 4 years. Serum FFA levels were calculated by comparing the amounts of

TABLE 1. AUTOMATIC AND MANUAL TITRATION OF SERUM EXTRACTS

Serum	FFA 1 Automatic a	1 Automatic b	1 Automatic Diff.	FFA 2 Manual a	2 Manual b	2 Manual Diff.	3 Average Values Auto	3 Average Values Manual	3 Average Values Auto Minus Manual
				($\mu Eq/liter$)					
1	321	342	21	322	329	7	331	326	5
2	542	548	6	549	534	15	545	543	2
3	495	479	16	472	479	7	487	477	10
4	495	484	11	531	531	0	489	531	−42
5	453	469	16	439	494	55	461	467	−6
6	390	390	0	348	370	22	390	360	30
7	362	344	18	385	385	0	353	385	−32
8	415	469	54	439	457	18	442	449	−7
9	285	285	0	293	256	37	285	275	10
Average			16 ± 16*			18 ± 18*			−3 ± 22†

Serum was obtained from 9 healthy volunteers, 2 females, 7 males, aged 16 to 34, after a 10–14 hr fast. Aliquots were frozen and at two different times thawed and extracted for either the automatic or manual titration procedure. a, b: duplicate determinations.

* Standard deviation.

† $t = 0.68$ (not significant).

NaOH used in titrating extracts of serum and standard palmitic acid, and the results obtained by each method were compared.

RESULTS

A linear relationship existed between the amount of standard palmitic acid present and the amount of titrant added (Fig. 2). The average difference in the amount of NaOH added to duplicate standards was the same for 445 and 890 μEq/liter. The difference between duplicates, expressed as a percentage of the total, averaged 1.6 for samples with 445 μEq/liter and 0.7 for samples with 890 μEq/liter. Thus, the difference between duplicate titrations of standards was extremely small. Owing to a constant error of the method, the percentage difference between duplicates increased as the sample size decreased.

Nine sera were extracted and titrated automatically (Table 1, column 1). The average difference between duplicate determinations on each serum was 16 ± 16 (SD) μEq/liter; in other words in 95% of the determinations the difference between duplicates was no greater than 48 μEq/liter. Results of the extraction and manual titration of the same nine sera are recorded in Table 1, column 2. The average difference between duplicate determinations, 18 ± 18 (SD), was virtually identical with that in the automatic titration. No significant difference between the mean values obtained by the two methods was found (Table 1, column 3).

Duplicate determinations by automatic titration were performed on 50 additional sera. The difference between duplicates was similar to that recorded in Table 1.

DISCUSSION

The relatively nonpolar single-phase system employed presented certain features not generally encountered with an aqueous medium, namely, (*a*) an initially high titration value for blanks, (*b*) a slow drift of the pH meter which appeared to be related to a tendency for blanks and small FFA samples to be overtitrated, and (*c*) a tendency toward fluctuations of the pH meter.

The initially high titration value was eliminated by soaking the electrode in a blank solution prior to any titrations. Overtitration of blanks and of small FFA samples was eliminated by allowing the drift of the pH meter to proceed before beginning the titration, and by titrating at a slow rate. The latter was accomplished by setting the instrument at a proportional band of 5, causing it to add small increments of titrant from the beginning of the titration, and thereby increasing the titration time. Under these conditions an average of 5 μl of titrant was added to the blanks. The following factors have eliminated fluctuations of the pH meter in most instances: (*a*) adequate grounding of the apparatus, (*b*) slow delivery of the nitrogen through a delivery tip that does not touch the electrode, and (*c*) the presence of thymol blue indicator.

The stabilizing property of thymol blue indicator is an interesting phenomenon. Since the indicator was made with NaOH, HCl, and ethanol, NaCl and ethanol were in effect added to the system along with thymol blue. Neither NaCl nor ethanol by itself afforded the same stabilizing influence as the complete indicator. The thymol blue also provided an indication of the rare occasions when overtitration occurred owing to inadequate stirring or too rapid titration.

The linearity (Fig. 2) and reproducibility in titrating

samples of standard palmitic acid attest to the reliability of the titration procedure. Table 1 illustrates that the automatic and manual titration methods gave similar results for each serum, and that the variability in duplicate determinations was similar. Previous studies (3) and the present study demonstrated no significant change in FFA values after 1 month of freezing.

The automatic titration is less tedious than the manual titration, and can be accomplished with less practice. It is relatively simple and in general requires only 2–4 minutes to perform a titration.

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