Automatic titration of plasma fatty acids by photocolorimetry

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ABSTRACT A photocolorimeter for rapid automatic titration of free fatty acids is described. The solvents, absolute ethanol and hexane, form a single-phase titration mixture containing Nile blue indicator. The titrant, NaOH in 60% ethanol, is delivered by a motor-driven microsyringe; as alkali is added and the titration mixture turns pink, the intensity of light reaching a photocell through a 600 mμ interference filter increases. Increased current is generated until at a preselected number of microamperes a cut-off switch is activated which halts the drive motor.

Titrations of FFA in the range 150–1500 μeq/liter of palmitic acid standard are accomplished in approximately 1 min with a standard error of the mean of ±1.3–6.5 μeq/liter. The titration end point is independent of the operator. The solutions are stable and the daily titration blank and calibration remain constant. The procedure, therefore, is relatively simple and is quickly set up for routine determinations.

KEY WORDS automatic · photocolorimeter · titration · free fatty acids · single phase · Nile blue

THE AUTOMATIC TITRATOR described here was developed for large numbers of determinations of plasma free fatty acids. The instrument is basically a photocolorimeter employing a filtered light source and utilizing the change in color of Nile blue indicator from blue in the presence of (fatty) acids to pink in the presence of alkali. The blue light emerging from the initially acid titrating solution is prevented from reaching a photocell by the interposition of a 600 mμ pink interference filter; as alkali is added red light is transmitted through the filter in increasing intensity and causes the photocell to put out increasing current. At a preselected number of microamperes a cut-off switch is activated which halts the motor-driven syringe delivering the alkali.

Abbreviation: FFA, free fatty acid.

METHOD

Reagents and Standards

Ethanolic NaOH. In order to maintain a single phase during titration, we used 0.01 and 0.02 N NaOH prepared in 55–60% ethanol. To 20 ml of distilled water in a 50 ml volumetric flask, 0.5 or 1.0 ml of 1 N NaOH was added and the flask was filled to the mark with 95% ethanol. The microsyringes we employed delivered 0.2 or 0.5 μl per micrometer dial division; thus, depending upon the normality of the alkali and upon the microsyringe selected, each scale division on the micrometer dial was equivalent to 2, 4, 5, or 10 mμeq of alkali.

Nile Blue Indicator Solution. The indicator (Nile blue A, National Aniline Division, Allied Chemical Corp., New York, N.Y.), 15 mg, was dissolved in 100 ml of absolute ethanol. After the addition of several drops of 1 N NaOH, the red solution should read 0.260 ± 0.010 OD units in the Beckman DU spectrophotometer at a wavelength of 600 mμ with a 10 mm light path. The excess alkali was removed by adding dropwise a 0.1 N acid until the indicator solution became a dark plum color and had a titratable acidity (blank value) of not more than 200 mμeq when 2 ml was titrated in 3 ml of hexane.

Standard Stock Solutions. A standard stock solution of palmitic acid, 51.28 mg/200 ml of hexane, contained 1.0 meq/liter. Working standards containing 150–1500 mμeq/3 ml of hexane were prepared by diluting, at 5 ml intervals, 5–50 ml of the stock standard to 100 ml with hexane.

Procedure

One milliliter of plasma was obtained from normally fed human subjects and from normal and hyperlipemic subjects after intravenous heparin injection. The FFA were extracted according to the method of Dole (1) except that hexane was substituted for heptane; this modification intensifies the color end point. (The Dole procedure pro-

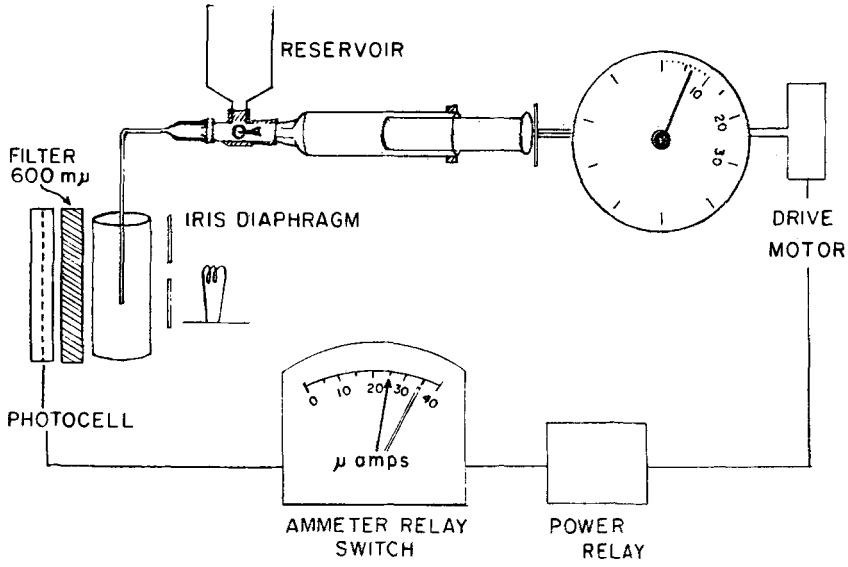

FIG. 1. Schematic diagram of the photocolorimeter shows the photovoltaic cell, (G.E. No. 8PV-1, General Electric Company, Schenectady, N.Y.) being energized by red light transmitted through the 600 mμ interference filter (B & L No. 44-7-60, Bausch & Lomb Incorporated, Rochester, N.Y.). The milliampere output is applied without amplification to the meter relay (A.P.I. No. 261, Assembly Products, Inc., Chesterland, Ohio), which acts as a cut-off switch to the power relay. The latter is activated by a line-operated d-c source and is used to control the electric clutch of the drive motor (Bristol Motors, Division of Volcaine Co. of America, Inc. Saybrook, Conn.) to the alkali delivery system (Syringe Microburette No. SB2, Micro-Metric Instrument Co., Cleveland, Ohio).

A list of specifications and parts numbers may be obtained from the author.

vides a 4 ml hexane extract for each milliliter of plasma extracted.) Titrations were then carried out in the automatic titrator as follows. Three milliliters of plasma hexane extract, or of a standard working solution of palmitic acid in hexane, were placed together with 2 ml of the Nile blue solution in a 2 × 4 cm (I.D. × height) flat-bottomed cylindrical vial containing a Teflon-coated stirring magnet. The vial was placed on a movable platform, which was raised into a darkened chamber 6 cm square and 10 cm high and brought the vial into the light beam. This maneuver also served to immerse the needle delivering the alkali below the level of the Nile blue–hexane mixture (Fig. 1).

The titratable acidity of the Nile blue solution, i.e. the daily titration blank, is obtained as follows. A vial containing 2 ml of the Nile blue solution and 3 ml of hexane is positioned in the titrator. The magnetic stirring motor is turned on at 300 rpm. The iris diaphragm aperture is reduced to about its midpoint before titration of the blank begins. This diminishes the intensity of the light source and thereby reduces the output of the photocell. The titration of the blank is performed manually, by adding five scale divisions of the alkali at a time until the maximum deflection of the microampere needle is observed. If this needle moves off the scale before the end point is reached, the iris diaphragm is closed down further and the titration continued. The titration blank remains constant for many months in each batch of Nile blue solution.

Calibration of the titrator for automatic operation is illustrated by the microampere titration curves shown in Fig. 2. The increase in the photocell output during titration, plotted on the vertical axis, is compared to the amount of alkali added in increments of 10 scale divisions. In this example it was calculated that a palmitic acid standard solution containing 600 mμeq/liter required 150 scale divisions of alkali (plus the blank value) when a syringe–alkali combination delivering 4 mμeq per scale division was used.

After the addition of 150 scale divisions of alkali, the photocell output was adjusted arbitrarily (see Discussion) to 35 μamp by opening or closing the iris diaphragm. The results of a slow manual titration are shown by the solid line. Without any change in the setting of the diaphragm, the same working standard was titrated rapidly by the motor-driven microsyringe to give the curve depicted with crosses. The difference between the two curves illustrates the time delay in color change during the more rapid addition of alkali. The iris diaphragm was then opened to increase the output from the photocell; the resultant curve for fast, automatic titration is shown by the open circles. It can be seen that this

latter curve intercepts the 150 scale division ordinate at 37 μamp. This, then, is the point at which the adjustable cut-off switch should be set for precise calibration.

It should be noted, however, that this degree of accuracy in calibration is not required, for a proportionate correction can be applied by observing the ratio of the calculated to the observed number of scale divisions on the micrometer dial after the blank value has been subtracted from the observed reading. Fig. 3 demonstrates that this proportionality is a straight line from zero intercept, with exceptions as noted.

The titrator may be calibrated by using oxalic in place of palmitic acid. One milliliter of 0.1 N aqueous oxalic acid is made up to 10 ml with 95% ethanol; 50, 100, or 150 μl is rinsed into 2 ml of the alcoholic Nile blue with a micropipette before the addition of 3 ml of hexane.

RESULTS

The results of analyses of working standard solutions of palmitic acid and of human plasma under the various conditions outlined are shown in Tables 1 and 2. It can be seen from the recovery data, standard deviation, and percentage errors in Table 1 that the results of this automatic titration procedure are more than adequately acceptable. It is noteworthy that the standard deviations in the titration of extracts of human plasma shown in Table 2 are approximately twice those found in the titration of the working standards. These differences are probably a measure of the handling and pipetting errors occurring during the extraction procedure.

Table 3 compares the results of titration of 16 replicates of the palmitic acid standards performed by the photocolorimeter with those executed by three experienced technicians. From the values for standard deviation it can be seen that technicians and instrument performed with comparable reproducibility. The photocolorimeter was twice as fast. The differences in values for percentage recovery reflect the difference in the end point chosen by each technician, as will be discussed below.

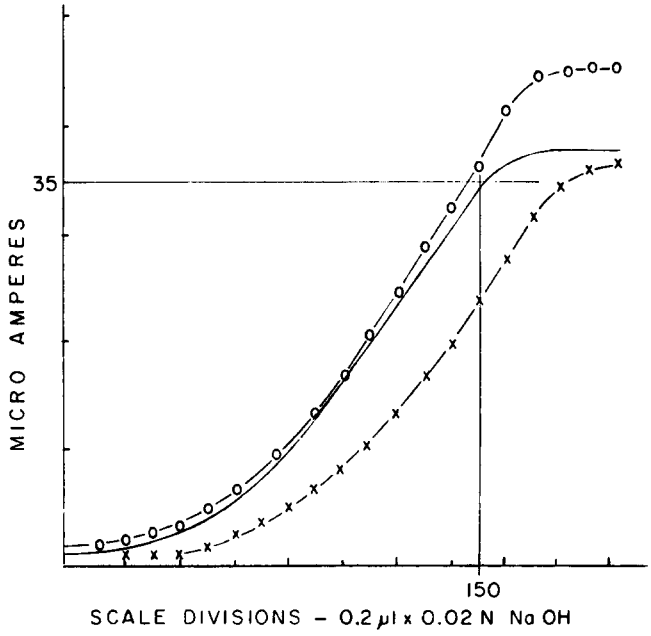

FIG. 2. As the Nile blue solution turns from blue to pink, increasing quantities of transmitted pink light reach the photocell which generates more current. The solid curve results from a slow manual addition of titrant; the ×——× curve derives from faster, motor-driven addition. The difference indicates the time delay in color change. The O——O curve is obtained by opening the iris diaphragm in front of the light source (Fig. 1) to compensate for the delayed color change, so that the cut-off switch is activated at, or close to, the number of microamperes corresponding to the number of millimicroequivalents of fatty acid. For clarity of presentation in this illustration and in the text, the blank value has been omitted from the calculations.

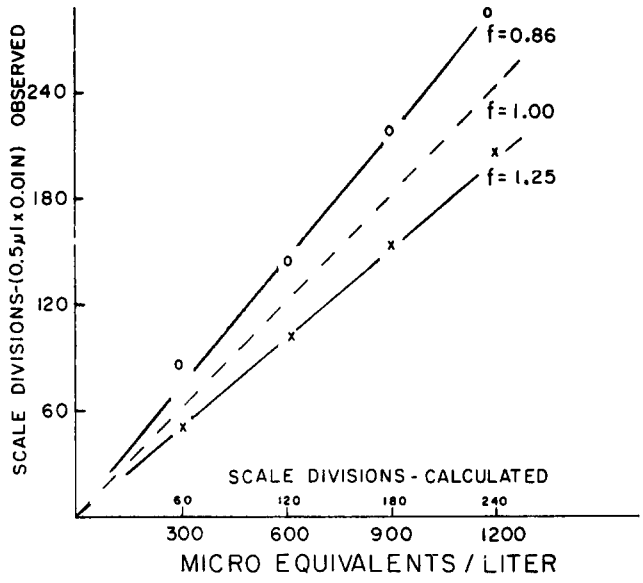

FIG. 3. Precise adjustment of the titrator is not required since a proportionate correction factor, *f*, can be applied to the number of millimicroequivalents observed. The proportion is obtained from the ratio of the calculated to the observed number of scale divisions on the micrometer dial for a standard fatty acid solution. The values used here to describe this ratio were extremes, and should not be duplicated in actual practice. Factors less than 1.00 result from diminishing the amount of light falling on the vial. Note the open circle corresponding to 60 scale divisions, calculated. The observed number of scale divisions is greater than calculated because the optical density of the Nile blue solution remained relatively undiluted by the small quantity of colorless titrant added. This has the same effect as decreasing the amount of light from the source.

DISCUSSION

Among the advantages of this titrator are its simple unamplified circuitry, its low cost, and the speed with which the analyses can be performed. The reagents are simply

TABLE 1 ANALYSES OF STANDARD SOLUTIONS OF PALMITIC ACID WITH DIFFERENT SYRINGE SIZES AND NORMALITIES OF TITRANT

Syringe Size and Normality of Alkali	Amount of Alkali per Dial Division		Concentrations of Standard Palmitic Acid Solutions (μeq/liter)						
			150	300	450	600	900	1200	1500
	mμeq								
0.2 μl × 0.01 N	2	% recovery	100.8	96.1	102.6	98.6			
		SD	10.7	11.5	12.9	10.9			
		% error	7.1	3.8	2.8	1.8			
0.2 μl × 0.02 N	4	% recovery		99.0	99.2	99.5	97.1	98.2	
		SD		11.7	12.6	16.0	12.2	26.1	
		% error		3.9	3.1	2.7	1.4	2.2	
0.5 μl × 0.01 N	5	% recovery		96.9	98.1	101.1	101.4	98.2	
		SD		12.0	12.3	5.2	18.9	18.4	
		% error		4.0	2.7	1.0	2.1	1.5	
0.5 μl × 0.02 N	10	% recovery		100.3		96.8	101.3	100.0	100.1
		SD		15.6		18.9	16.8	24.7	26.6
		% error		5.2		3.2	1.9	2.1	1.8

SD, standard deviation (n = 16); % error, SD/μeq of FFA.

The measured concentration of palmitic acid was calculated with the use of the calibration factor (Fig. 3). This accounts for the recovery's being occasionally greater than 100%.

TABLE 2 FFA ANALYSES (16 REPLICATES) OF HUMAN PLASMA OBTAINED FROM DIFFERENT SOURCES AFTER VARIOUS PROCEDURES

Conditions of Collection	Amount of Alkali per Dial Div.	Mean Value ± SD
	mμeq	*μeq/liter*
After 50 g of glucose	2	369.5 ± 20.0
Postprandial	2	478.1 ± 15.1
Pooled fresh serum	5	759.8 ± 26.0
Postprandial	5	895.8 ± 29.9
30 Min after heparin i.v.	10	1066.1 ± 41.6
10 Min after heparin i.v.	10	1448.7 ± 48.1
Pooled frozen serum	5	1488.6 ± 49.5
10 Min after heparin i.v.	10	2327.8 ± 89.0
Palmitic acid working standard 2400 μeq/liter	10	2322.0 ± 42.1

TABLE 3 COMPARISON OF THE MANUAL TITRATION OF PALMITIC ACID STANDARDS AND TITRATION BY THE AUTOMATIC PHOTOCOLORIMETER

	Technician			
	1	2	3	Automatic
% Recovery	90.2	97.7	102.2	99.6
SD (n = 16)	13.3	11.8	10.6	12.6
Minutes required for 16 samples	38	32	28	15

prepared and last indefinitely without refrigeration, with the exception of the palmitic acid standard solutions.

The adjustment of the size of the iris diaphragm at the light source determines the setting of the cut-off switch, but the aperture size is not critical. Referring to Fig. 2: where the syringe–alkali combination delivered 4 mμeq per scale division, it was calculated that 150 micrometer scale divisions were needed to titrate a palmitic acid standard containing 600 μeq/liter. In this illustration the diaphragm aperture was set at 10 units and the cut-off was adjusted to 37 μamp. A reduction in aperture size to 4 units required that the cut-off switch be set at 9 μamp for the delivery of the same quantity of alkali. Between these two settings a family of an infinite number of curves can be observed.

The optical density of the Nile blue solution is somewhat critical when the correction factor, Fig. 3, is to be applied over a wide range of values of FFA. When the indicator is concentrated, it will absorb light even at neutrality and thus cause overestimation of small quantities of FFA. On the other hand, when large quantities of FFA are titrated, the diluting effect of the colorless titrant will lead to underestimation of the FFA. The optimum concentration of the Nile blue seems to be that which gives 0.25–0.27 OD units.

The comparison between the manual and the automatic titration techniques, Table 3, brings out an additional point of interest. Not only are the manual titrations tedious and time-consuming, but they are further limited in that one technician must make all determinations in any series of related measurements. We have found that the color end point chosen by each technician is different. The automatic titrator, once set, has, of course, a constant end point which is independent of the operator.

From Table 1 it can be seen that the syringe size and the normality of the titrant are not critical. Where FFA are expected to be elevated, much time is saved and accuracy is not sacrificed if the large combination is used; for very high values of FFA, 0.02 N alkali should be used

if the appearance of a two-phase system is to be avoided.

The titrator operates satisfactorily in the titration of inorganic alkaline solutions with an acid titrant. The procedure remains unchanged except that the 600 mμ filter is replaced by a 500 mμ filter.

Three other systems for the automatic determination of plasma FFA have been described. Antonis (2) adapted the copper diethyl dithiocarbamate method of Duncombe to the Technicon Autoanalyzer. FFA were determined on duplicate pairs of 20 plasma specimens. The mean difference between duplicate pairs was 12 μeq/liter, with a standard error of a single determination of ±10.5 μeq/liter for the range 220–1300 μeq/liter of plasma FFA. Thirty samples can be analyzed in 1 hr. The preparation of the phospholipid-free chloroform extract consumes more time than the Dole extraction procedure. Schnatz (3) titrated FFA in heptane with NaOH using the Radiometer TTTlc titrator. Nine sera were extracted by the Dole procedure and titrated automatically. The average difference between duplicate determinations on each serum was 16 ± 16 μeq/liter. Each titration required 2–4 min. Kelley (4) has described a method of automated microtitration of fatty acids which is similar in principle to the one described here. A single-phase system is maintained by the use of absolute ethanol and heptane, with tetrabutyl ammonium hydroxide in methanol as the titrant and phenol red as the indicator. The titrations are carried out in the well of a colorimeter (Bausch & Lomb Spectronic 20). The output from the colorimeter is connected to a meter relay which governs the titrant delivery motor. Four replicate titrations of oleic acid standards, 200–1200 μeq/liter, agreed to within ±2.5% of their mean. Our initial investigations with titration curves were performed with a colorimeter. It was found more convenient, however, to construct an inexpensive self-contained unit.

For the automatic titrations of FFA described here, in the range 150–1500 μeq/liter of palmitic acid standards the standard error of the mean (16 replicates) was between 1.3 and 6.7 μeq/liter. Titrations were performed in approximately 1 min. The titration end point is independent of the operator. The solutions are stable, the daily titration blank and calibration remain constant. The procedure, therefore, is relatively simple and is quickly set up for routine determinations.

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