Modified semiautomated method for free fatty acids in serum

BRENDA CRANE AND CHRISTINE LANE

From Ruakura Agriculture Research Centre, Ministry of Agriculture and Fisheries, Private Bag, Hamilton, New Zealand

SUMMARY Modifications have been made to the reagent system of the semiautomated method for the determination of plasma free fatty acids by Baird *et al.* (1967), and results are reported of investigations into parameters affecting sensitivity and analytical range.

Semiautomatic methods described for the determination of free fatty acids in blood plasma involve the conversion of phenol red to its yellow acid form after the release of free diethylbarbituric acid from the sodium salt (sodium barbitone) by fatty acids (Mosinger 1965; Baird *et al.* 1967). The method of Baird *et al.* (1967) includes the use of a range expander in the automated system to increase the sensitivity of the recording. The present paper reports a method of obtaining similar sensitivity without a range expander by adjusting the ratio of buffered phenol red to sodium barbitone and selecting the optimal baseline level. Using extracts prepared as recommended by Baird *et al.* (1967), the accuracy of the AutoAnalyzer method was assessed against Annison's (1960) titrimetric method in which Nile blue with its more easily defined end point, replaces the thymol blue indicator of Dole's (1956) established method.

Material and methods

REAGENTS

Extraction mixture

Isopropanol 40 parts; heptane, 10 parts; 0.5 M (1N) sulphuric acid 1 part.

Buffered phenol red stock

1% solution of phenol red in 2.5% sodium barbitone.

Sodium barbitone stock

2.5% sodium barbitone (0.25 g in 10 ml water).

PHENOL RED WORKING SOLUTIONS

These were prepared one hour before use.

The volumes of stock solution indicated were diluted to 100 ml with ethanol.

Solution	Stock phenol red (ml)	Stock sodium barbitone (ml)	Final concentration sodium barbitone %
1	0.2	—	0.0050
2	0.3	—	0.0075
3	0.4	—	0.0100
4	0.5	—	0.0125
5	0.3	0.03	0.00835
6	0.3	0.07	0.00925
7	0.3	0.10	0.01000
8	0.3	0.15	0.01125
9	0.3	0.20	0.01250
10	0.4	0.01	0.01025
11	0.4	0.03	0.01075
12	0.4	0.05	0.01125
13	0.4	0.07	0.01175

PALMITIC ACID¹ STANDARDS

Standards ranging from 160 to 1440 $\mu\text{mol/l}$ ($\mu\text{Eq/l}$) were prepared in heptane and extracted according to Baird *et al.* (1967). Free fatty acids from both standards and plasma were extracted into heptane by the same method. Standards or plasma (1 ml) were mixed in test tubes with extraction mixture (5 ml), heptane (3 ml) and boiled distilled water (2 ml) and shaken vigorously for 1½ minutes. Heptane saturated nitrogen was bubbled through each tube to remove volatile acids (especially carbon dioxide) which are present in greater amounts in plasma than in standards. The heptane phase was washed once with 4 ml 0.05% v/v sulphuric acid to remove lactic acid and an acetone insoluble lipid—probably phospholipid (Baird *et al.*, 1967)—and the extracts were stored at 4°C until analysis. Baird found plasma extracts stable for at least three days when stored at 4°C.

¹For palmitic acid, $\mu\text{mol/l} = \mu\text{Eq/l}$
 $\mu\text{mol/l} \times 0.02565 = \text{mg/100 ml}$

AUTOANALYSIS

The manifold was the same as that described by Baird *et al.* (1967). A stream of working phenol red reagent was segmented with carbon dioxide-free air. After a steady baseline had been established the sample was introduced into the stream which was passed through a double mixing coil, the air was eliminated and the decrease of the red colour was recorded at 550 nm.

ANNISON'S METHOD

For Annison's titrimetric method all sera and standards were extracted as for autoanalysis (Baird *et al.*, 1967). Two millilitres of the extract were evaporated to dryness under nitrogen; the residue was dissolved in 1 ml ethanol (95 %) and titrated with 0.01 M sodium hydroxide under a stream of nitrogen, 1 ml of 0.002 % Nile blue in 95 % ethanol being used as indicator.

Experimental

The following trials were carried out to study the influence of various factors on sensitivity:

VARIATION OF INDICATOR CONCENTRATION

To establish the maximum sensitivity obtained with varying concentrations of buffered phenol red solutions without additional sodium barbitone, standards containing from 160 to 1440 $\mu\text{mol/l}$ palmitic acid were run on the AutoAnalyzer with working concentrations of phenol red ranging from 0.002 % to 0.005 % containing sodium barbitone in concentrations ranging from 0.005 % to 0.0125 % (working solutions 1-4). The reagent baseline was set at 10 % T at the beginning of each run.

Sensitivity increased significantly as the concentrations of the phenol red and plamitic acid solutions were increased, the maximum being attained with 0.005 % phenol red solution (working solution 4) and the 1120 $\mu\text{mol/l}$ standard which gave 81 % T, the recording being off scale with 1280 $\mu\text{mol/l}$ (Table 1a). Because the colour decreases as the concentration of plamitic acid increases the baseline is set at low transmission. Sensitivity is poor in this region.

Consequently, sensitivity is poor with low concentrations of acid and increases progressively with increased concentrations.

VARIATION OF BUFFER CONCENTRATION

To establish the optimal concentration of sodium barbitone relative to that of phenol red, standards were run with working solutions containing both 0.003 % phenol red and sodium barbitone ranging from 0.00925 to 0.0125 % (working solutions 6 to 9), and 0.004 % phenol red and sodium barbitone ranging from 0.01025 to 0.01175 % (working solutions 10 to 13).

The results are given in Table 1b. With the 0.003 % phenol red sensitivity was greatest for all palmitic acid concentrations when the sodium barbitone concentration was of 0.0100 % (working solution 7) but was reduced at sodium barbitone concentrations of 0.01125 % (working solution 8) and above. With 0.004 % phenol red sensitivity was greatest with 0.01025 % sodium barbitone (working solution 10) for palmitic acid concentrations of 800 $\mu\text{mol/l}$ and above. With 0.01075 % sodium barbitone (working solution 11) sensativity was greatest for all palmitic acid concentrations up to 640 $\mu\text{mol/l}$ but was least for all palmitic acid concentrations from 800 to 1440 $\mu\text{mol/l}$. With greater concentrations of sodium barbitone sensitivity was reduced for palmitic acid solutions between 160 and 640 $\mu\text{mol/l}$ but was increased significantly from 800 to the maximum concentration of 1440 $\mu\text{mol/l}$ (Table 1b).

VARIATION OF BASELINE SETTING

Variation of sensitivity with baseline levels set between 5 % and 20 % T were examined using 0.003 % phenol red in 0.00825 % sodium barbitone (working solution 5) and 0.004 % phenol red in 0.01075 % sodium barbitone (working solution 11). These less sensitive barbitone concentrations were chosen because they accommodate a wide range of standards and a high transmission baseline.

Sensitivity increased significantly with every 5 % T rise in baseline regardless of the reagent concentrations. The 0.003 % phenol red with 0.00825 % sodium barbitone gave maximum sensitivity at 15 % T

Table 1a *Effect of phenol red concentration on sensitivity (change in % transmission for 160 $\mu\text{mol/l}$ palmitic acid) with the baseline at 10 % T*

% Phenol red % Barbitone		Change in % T								
		0-160	160-320	320-480	480-640	640-800	800-960	960-1120	1120-1280	1280-1440
0.002	0.005	4	3	4	4.5	3	2	1	1.5	0.8
0.003	0.0075	3	3	3.5	4.5	5	5	5	3.8	2
0.004	0.0100	4	4	6	7.8	10	12.2	18.2	22	off scale
0.005	0.0125	4.8	4.2	6	7.2	11.8	16	21.5	off scale	off scale

Table 1b *Effect of sodium barbitone concentration on sensitivity (change in % transmission for 160 μ mol/l palmitic acid with the baseline at 10% T)*

% Phenol red % Barbitone		Change in % T								
		0-160	160-320	320-480	480-640	640-800	800-960	960-1120	1120-1280	1280-1440
0.003	0.00925	1.8	2.2	3	3.2	4	5.5	6.5	8	10
	0.0100	3	3.2	4.3	4.7	5.6	6.5	8	8.5	10.8
	0.01125	2	3	3.2	3.8	4.8	5.2	6	6	7.5
	0.01250	2	3	3	3.7	4.5	4.8	4.8	6	6.5
0.004	0.0100	3	4.8	7	8	10.5	12.5	15	20	off scale
	0.01025	3	4.5	6	7.5	11	14	16	20	off scale
	0.01075	5	5.5	7	8	10	11.2	13.2	15	15.2
	0.01125	3	4	5.8	7.2	11	14	16	18.8	off scale
	0.01175	3	3.5	5.5	6.5	8.5	11.5	16	18.7	off scale

baseline with the full range of standards on scale. Using 0.004% phenol red and 0.01075% sodium barbitone the full range of standards was on scale with the baseline set at 10% T; at 15% T the range was reduced to 960 μ mol/l with increased sensitivity, and at 20% T maximum sensitivity was achieved but with the range reduced to 800 μ mol/l (Fig. 1). To obtain 20% T with this high optical density reagent, it was necessary to reduce the aperture on the reference side to less than the 6 mm diameter of the standard Technicon AutoAnalyzer I aperture No 1. A metal plate with a 4 mm hole was used.

COMPARISON OF METHODS

To show the comparability of the AutoAnalyzer method with that of Annison, ovine sera with a wide range of values were analysed in duplicate by both methods (Fig. 2) (Table 2).

Variation between duplicates was less for the AutoAnalyzer method than for Annison's, but in both cases it increased with increasing fatty acid concentration so that the coefficients of variation were stable for the two methods, averaging 2.5% and 5% respectively. Because of this non-homogeneity of variance, regression analysis was carried out with logarithmically transformed data. This gave no statistical evidence that the relationship between AutoAnalyzer and Annison estimates differed between the sets, leading to the equation for sample means: $\log_e M = \log_e AA + 0.0181$ (residual SD = 0.054). This is equivalent to $M = 1.018AA$ with a residual error of 6%.

Determinations by the AutoAnalyzer on bovine sera were not subject to interference by carotenoids which affect indicator changes in the manual titration.

Fig. 1 *Variation of sensitivity with the reagent baseline set at 5, 10, 15, and 20% T. Reagent 0.004% phenol red and 0.01075% sodium barbitone.*

Table 2 Standard deviations within duplicate determinations by the AutoAnalyzer and Annison's (manual) methods

Data Set	No. of samples	No. of determinations	AutoAnalyzer		Manual	
			Mean	SD	Mean	SD
I	13	26	351	8	360	15
II	5	10	495	14	522	32
III	8	16	1228	31	1220	71

Fig. 2 Regression of serum free fatty acid levels obtained by AutoAnalyzer against those obtained on the same specimens by Annison's titrimetric method. The line shown is the 1:1 relationship.

Discussion

From examination of Tables 1a and 1b it is evident that the sensitivity of the colour reaction is attributable to the phenol red. Sodium barbitone is needed as a buffer at a minimal concentration of 0.01%. Higher concentrations tend on the whole to inhibit the reaction.

From the above data it is possible to select the

optimal ratios of stock phenol red to stock sodium barbitone, and the optimal baseline level to cover either a wide range of fatty acid concentrations or the maximal sensitivity for a selected narrow range of concentrations. It appears that a reagent consisting of 0.004% phenol red and 0.01075% sodium barbitone (working solution 11) at 10% T baseline gave the optimal sensitivity for fatty acid values between 160 and 1440 $\mu\text{mol/litre}$. To obtain greater sensitivity at the lower values it is necessary to select a higher baseline. Conversely to obtain values above 1440 $\mu\text{mol/l}$ it is necessary to select a lower baseline.

Day-to-day inconsistencies were experienced at baseline 5% T and below, which we attributed to insufficient light passing through the colorimeter to give sufficient impulse for adequate response from the recorder.

We thank Dr E. Payne and Mr N. T. Clare for helpful advice, Mr D. M. Duganzich for the statistical analyses, and Mr P. M. Dobbie for collecting the samples.

References

- Annison, E. F. (1960). Plasma non-esterified fatty acids in sheep. *Australian Journal of Agricultural Research*, **11**, 58-64.
- Baird, J. D., Black, M. W., and Faulkner, D. E. (1967). Semiautomated method for the determination of free fatty acids in plasma. *Journal of Clinical Pathology*, **20**, 905-909.
- Dole, V. P. (1956). A relation between non-esterified fatty acids in plasma and the metabolism of glucose. *Journal of Clinical Investigation*, **35**, 150-154.
- Mosinger, F. (1965). Photometric adaptation of Dole's microdetermination of free fatty acids. *Journal of Lipid Research*, **6**, 157-159.