Colorimetric Method for Free Fatty Acids in Serum Validated by Comparison with Gas Chromatography

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We compared results by a simplified colorimetric copper-soap method for serum free fatty acids (Clin. Chem. 19: 419, 1973, modified) with those by a gas–liquid chromatographic method. The modified method requires only 100 µL of sample, its standard curve is linear from 0.1 to 4.0 mmol/L (reference interval for adults: 0.2 to 0.8 mmol/L), and it is suitable for use with newborns. Comparison with the gas–liquid chromatographic method (n = 51) over a wide concentration range gave a correlation coefficient of 0.989. Between-run CVs varied from 4 to 10%, analytical recoveries from 97 to 104%. Triglycerides, salicylates, hemoglobin, and anticoagulants (except citrate) do not interfere, but bilirubin and phospholipids give small positive interferences. The colorimetric assay is used, along with other routine tests, to assess lipid status and estimate kernicterus risk in neonatal intensive-care patients receiving lipid nutrition parenterally.

Additional Keyphrases: kernicterus risk in newborns • parenteral nutrition with lipid • reference intervals

Recently, measurement of serum free fatty acids (FFA) have been used to help assess kernicterus risk in premature infants. Many of these infants receive lipid diets parenterally (1–3). The resulting increase in FFA is known to displace bilirubin from albumin binding sites, which is believed to increase the risk of kernicterus (4). This risk is enhanced when total bilirubin concentrations are increased and albumin concentrations and blood pH are low (1).

Methods for FFA analysis include titration (5, 6), colorimetry (7–12), radioassay (13), and gas-chromatographic techniques (14–16). Of these, only the colorimetric and radioassay methods meet the requirements for a small sample volume, but the radioassay methods require specialized equipment and radioisotope handling. In contrast, the colorimetric copper-soap methods are inexpensive, convenient, and reliable. Soloni and Sardina (7) proposed such a method for use with adult sera. In this method, which is similar in principle to that of Duncombe (8), serum is mixed with buffered Cu²⁺ solution and chloroform. Copper ions react quantitatively with FFA to form a complex, which is extracted into the chloroform phase. Unreacted copper ions remaining in the aqueous phase are removed and an aliquot of the chloroform phase is mixed with a chromogenic reagent that is sensitive to copper. The absorbance of the colored product is measured at 620 nm. Various color reagents have been used (7–12), but cuprizone [oxalic acid bis(cyclohexylidenedenaryldrazide)] is highly satisfactory, owing to the high absorbptivity of its copper complexes.

We present modifications of the method of Soloni and Sardina, which simplify the procedure even further and extend the linear range for use with neonatal patients. We find that phase separation by simple aspiration and the use of easily prepared hexane-based standards are adequate for good quantitative work, whereas Soloni and Sardina used filter paper pads and standards prepared in an aqueous purified albumin matrix. We evaluate the effect of these modifications on method accuracy by comparison with results by a gas–liquid chromatographic method (14) for FFA concentrations ranging from those found in normal adults to values exceeding those expected in a neonatal population. In none of the published colorimetric methods (7–12) was gas–liquid chromatography used as a comparison method.

Materials and Methods

Apparatus

We used a Model 25 spectrophotometer (Beckman Instruments, Inc., Irvine, CA 92664) for all absorbance measurements. A vortex-type mixer with a 16-tube shaker head was used, as previously described (7). For the gas–liquid chromatographic method, we used a 5700 Series gas chromatograph, equipped with a flame ionization detector and a Model 3370B electronic integrator (all from Hewlett Packard, Avondale, PA 19311). A glass column, 1.83 m × 2 mm (i.d.), was packed with 10% Silar 10C on 100/120 mesh GasChrom Q (Applied Science Lab., State College, PA 16801). Oven, injection port, and detector temperatures were 160, 250, and 250 °C, respectively. Gas flow rates for N₂, H₂, and air were 30, 30, and 220 mL/min, respectively. Using this procedure, we quantitated the C₁₄ through C₂₀ fatty acids.

Reagents

Copper reagent, cuprizone reagent, and ammonia reagent used in the colorimetric method were prepared as described previously (7). For the gas-chromatographic method, the internal standard, methylating agent, and methyl propionate were obtained from Applied Science Lab. A 50 mg/L solution of the internal standard (n-pentadecanoic acid) was prepared in hexane (Burdick and Jackson, Muskegon, MI 49442). The methylating reagent, “Meth-Prep I,” a 0.2 mol/L aqueous solution of (m-trifluoromethylphenyl)trimethylammonium hydroxide, was used as supplied. Other reagents used were methyl propionate (1 part)/methanol (2 parts) and phosphoric acid (1.0 and 0.1 mol/L).

A 1.0 g/L FFA standard was prepared by dissolving palmitic acid (Sigma Chemical Co., St. Louis, MO 63178) in hexane. Standards of 500, 250, and 100 mg/L were prepared by appropriate dilution of the 1.0 g/L standard with hexane. Fresh standards were prepared weekly.

Procedure

Colorimetric method. Allow standards and samples to reach room temperature. To labeled 16 × 100 mm disposable tubes,
add 100 µL of serum, standard, or control. Use 100 µL of water for the blank tube. Add 300 µL of copper reagent and 2.0 mL of chloroform to each tube, including the blank tube. Stopper each tube and place the tubes in the shaker. Avoid contact of tube contents with the stopper. Shake for 10 min, then centrifuge. Carefully aspirate the (blue) aqueous layer, using a water-tap aspirator. Pipet 500 µL of each chloroform layer into labeled 13 × 100 mm disposable tubes. Wipe the pipette tip carefully before and after delivery. Add 1.0 mL of cuprizone reagent to each tube and shake gently. Add 100 µL of albumin reagent to each tube, stopper with a plastic cap, and shake briefly by hand. Ten minutes after adding the ammonia, read the absorbance at 620 nm of the contents of each tube vs the blank tube. The color is stable for 20–25 min. Construct a standard curve and read the concentration of each unknown from this curve. Convert mg/L to mmol/L as follows:

\[ \text{mmol/L} = \left( \frac{\text{mg/L}}{256} \right) \]

where 256 = rel. molecular mass of palmitic acid.

Gas-chromatographic method. To labeled 16 × 125 mm screw-capped tubes, add the following to each tube: internal standard (50 mg/L), 500 µL; sample, standard, or control, 500 µL; methanol, 1.5 mL; hexane, 5 mL; and 1 mol/L H₃PO₄, 500 µL. Shake the tubes for 1 min and centrifuge. Transfer the hexane phases to labeled 13 × 100 mm tubes. Wash the hexane phases twice with about 2 mL of a 0.1 mol/L solution of H₃PO₄. Centrifuge after each wash. Transfer the hexane phases to "nipple" extraction tubes. Slowly add 10 µL of Meth-Prep I to each tube while the tube is vortex-mixing, then centrifuge. Pre-wet a 10-µL syringe (Hamilton Co., Reno, NV 89510) with methyl propionate/methanol solution. Wipe the tip and draw up 2 µL of the Meth-Prep I (lower) phase. Wipe the syringe contents rapidly into the gas chromatograph. Calculate the concentration of each fatty acid as follows:

\[ \text{Concentration, mg/L} = (\text{peak area/peak area, internal std.}) \times \text{conc} \times \text{std.} \]

Convert mg/L for each FFA to mmol/L by dividing by the respective molecular masses, and add these values together to obtain the total FFA concentration.

Results

Our modifications of the colorimetric method (7) are: standards are prepared in organic solvent instead of aqueous solutions of purified albumin, phases are separated by simple aspiration instead of with filter paper pads, and we use a double-beam spectrophotometer for greater measurement accuracy.

Palmitic acid standards. We observed no difference between standard curves prepared from hexane-based standards (y = 0.921x – 0.010, Sₓᵧ = 0.007) and aqueous purified albumin standards (y = 0.917x – 0.013, Sₓᵧ = 0.006), where y milli-absorbance (m4) and x = concentration (mg/L).

Analytical recovery. Analytical recovery was evaluated by adding known amounts of palmitic acid to normal and pathological patient sera and assaying. Recoveries ranged from 97.1 to 104.1%, for 0.7 to 3.1 mmol added per liter.

Comparison with gas–liquid chromatography. Because sample-volume requirements of this method are so great, neonatal sera were not used for comparisons, but instead sera from normal adults, sera from unselected hospitalized patients, and sera supplemented with palmitic acid. The results show good agreement between the methods (n = 51, y = 0.926x + 0.032, r = 0.9887, SDslope = 0.020, SD_intercept = 0.027). The colorimetric method gives slightly lower results because stearic and C₂₀ acids are not extracted as efficiently as are palmitic, oleic, myristic, and linoleic acids (7).

Interferences. Triglycerides (1 g/L) and salicylates (500 mg/L) did not interfere with the assay. Hemoglobin and anticoagulants (except citrate) have also been shown not to interfere (7). The addition of 100 mg of bilirubin per liter resulted in an increase of 0.1 mmol/L in the reading for apparent FFA; 500 mg of phospholipid per liter caused an increase of 0.05 mmol/L. Apparently, these two substances are to some extent extracted as copper complexes. The presence of turbidity (by visual observation) had no effect on the results.

Reference interval. For 37 apparently healthy non-fasting adult blood donors (21 men, mean age 42 years, range 24–57; 16 women, mean age 31, range 18–59), the mean FFA concentration was 113 mg/L (0.44 mmol/L), with a range of 51–205 mg/L (0.2–0.8 mmol/L), as determined by the percentile method (17). Reference intervals for fasting adults as determined by various methods range from 24–204 mg/L to 116–238 mg/L (7). Fasting persons have higher plasma FFA concentrations than non-fasting persons. Due to the lack of published data for comparison and the fact that FFA concentrations in neonates who are receiving lipids intravenously can vary widely, normal values for this patient population were not determined.

Precision. Within-run and between-run precision (Table 1) was determined by use of normal sera, pathological sera, and supplemented sera (low values are not clinically significant).

Discussion

The colorimetric coppper soap method described here is well suited to the determination of FFA concentrations in serum from neonatal intensive-care patients. The modifications presented greatly simplify the assay without compromising accuracy, as illustrated by the comparison, recovery, and reference-interval studies. We have extended the linear range to 4 mmol/L (1000 mg/L), as compared to 500 mg/L (7), by using less of the chloroform extract (0.5 mL vs 2 mL) and more cuprizone reagent (1.0 mL vs 0.9 mL) in the color-development step. An upper linearity limit of 4 mmol/L (fivefold the upper limit of normal for adults) suffices for analyses of sera from newborns. Peak FFA concentrations are usually less than 4 mmol/L (3). Removal of the aqueous phase (dark blue) from the chloroform phase (colorless) by simple aspiration has not caused difficulties in our hands. However, we recommend the use of a vortex-type multiple shaker head and the reagent formulations advocated by Soloni and Sardina. We find it desirable to report results in units of both mg/L and mmol/L. The latter units are more useful in determining kernicterus risk, because the molar ratios of FFA, bilirubin, and albumin are the important criteria for clinical interpretation (1).

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Automated Discrete Kinetic Method for Erythrocyte Acetylcholinesterase and Plasma Cholinesterase

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We describe an automated kinetic method for erythrocyte acetylcholinesterase (EC 3.1.1.7) and plasma cholinesterase (EC 3.1.1.8) based on Ellman’s colorimetric method. Quinidine sulfate is used as an inhibitor of plasma cholinesterase during the measurement of erythrocyte acetylcholinesterase activity, obviating the need for washing the erythrocytes before lysis. Results by this method are compared with those obtained by the electrometric ΔpH method of Michel. To emphasize the need for measuring both erythrocyte acetylcholinesterase and plasma cholinesterase activity in workers exposed to organophosphate pesticides, we present a study of serial activities of both enzymes in a person accidentally exposed to demeton–S-methyl.

Additional Keyphrases: toxicity · organophosphate pesticides · environmental and occupational hazards · methods for the small laboratory

The increased incidence of human exposure to pesticides, occupationally or environmentally, has resulted in many requests for clinical laboratories to monitor such patients routinely. In many hospitals, exposure to organophosphate pesticides is monitored by measuring plasma cholinesterase (EC 3.1.1.8, acetylcholine acetylhydrolase) activity alone. However, the measurement of erythrocyte acetylcholinesterase (EC 3.1.1.7, acetylcholine acetylhydrolase) in conjunction with plasma cholinesterase activity is preferable (1, 2). Plasma cholinesterase activity is depressed in certain pathological and physiological conditions (3–5); genetically determined variants exist in which activity is decreased (6, 7); and plasma cholinesterase and erythrocyte acetylcholinesterase may be inhibited to different degrees and for differing lengths of time after exposure, depending on the organophosphate (8).

Methods currently in use for measuring erythrocyte acetylcholinesterase include electrometric ΔpH (9, 10) and pH-stat methods (11) and spectrometric methods involving various substrates (12, 13).

Here we describe and evaluate an automated kinetic assay for both the erythrocyte and plasma enzymes; the method is based on the colorimetric procedure of Ellman et al. (19), but does not require preliminary washing of the erythrocytes. The results from this automated method are compared with those from the electrometric ΔpH method of Michel (9).

Materials and Methods

Reagents

Acetylthiocholine iodide, 7.3 mmol/L. This solution may be stored for as long as two months at −18 °C.
5,5'-Dithiobis-2-nitrobenzoic acid (DTNB) stock solution,