V. ANALYTICAL METHODS

G. Quantitative Urinary Porphobilinogen Ion-Exchange Chromatography

1. Principle

Porphobilinogen (PBG), a precursor of porphyrins, is formed by the condensation of delta-aminolevulinic acid (D-ALA) in a series of reactions that eventually lead to heme. Porphyrin and heme biosynthetic activity takes place in all body cells, but is most prominent in the marrow of the long bones and in the liver.

Normally, only minute amounts of free porphyrins are present in the body but porphyrin levels are found to be increased in the urine as a result of vitamin deficiencies, liver damage (jaundice, infections, hepatitis, and cirrhosis), lead poisoning, certain drugs (arsenic, carbon tetrachloride, benzene, and sulfonamides), as well as in "inborn errors" of porphyrin metabolism.

In this assay, porphobilinogen is purified by absorption on an ion-exchange resin that removes color-producing substances, indole, and related compounds that react with the chromophore to produce colorless derivatives. After being eluted from the resin column with acetic acid, PBG is condensed with p-dimethylaminobenzaldehyde in acid (Ehrlich's reagent) to form a magenta-colored product.

2. Sample Collection and Storage

a. No participant preparation is necessary.

b. A well-mixed aliquot (8-20 ml) of a 12-hour urine collection is used for this test. Urine specimens are collected with 2.0 g sodium carbonate per 1,500 ml. The specimen should be protected from light and kept cold during collection.

c. The specimen is stable 24 hours at 2-8 °C. For longer storage, freeze at -20 °C.

3. Reagents, Supplies, and Equipment

a. Glass test tubes, disposable: 16 X 100 mm

b. Pipets: Repipettes, 4 ml, 5 ml, 25ml
Class A volumetric, 25 ml, 4 ml, 1 ml MLA, 1 ml, 500 ul with disposable tips
Accuprep (Beckman)
Macro MLA, 1-5 ml adjustable.
Eppendorf repeating pipet, 5 ml and 12.5 ml, disposable Combitips.

c. Volumetric flasks: 10 ml, 50 ml, 100 ml, 1 L. Class A

d. Analytical balance (Mettler AE 166)

e. Timer

f. Vortex mixer

g. Draining rack and waste container

h. Plastic transfer pipet

i. Funnels for columns (Whale Scientific)

j. Test-tube racks

k. Scissors or large nail clipper

l. pH meter, Fisher Accumet model no. 805

m. Spectrophotometer with 1- to 2-nm band width (Beckman Du-7)

n. Magnetic stirrer

o. Acetic Acid, 1M, (analytical grade, Mallinckrodt)

(1) Combine in a 1-L volumetric flask (work under fume hood):

(a) Glacial acetic acid	57 ml
(b) Reagent water type I	QS to 1 L

(2) Transfer to brown glass bottle with 4-ml repipet attachment. Label as "Acetic Acid 1M" with the date of preparation, technician's initials, and an expiration date of 1 month. Store at room temperature.

p. Phosphate Buffer, 0.056M, pH 7.6

(1) Solution 1: Potassium biphosphate 0.1 M (KH_2PO_4)
(a) Potassium phosphate 13.609 g, reagent grade
(b) Fisher reagent water type I, QS to 1 L

Dissolve in a 1-L volumetric flask, bottle, and label "Potassium biphosphate 0.1M" with date of preparation, technician's initials, and an expiration date of 1 year. Store at room temperature.

(2) Solution 2: Sodium hydroxide 0.1M (NaOH)
(a) Sodium hydroxide, 4 g reagent grade ACS, MCB
(b) Reagent water type I, QS to 1 L

Dissolve in a 1-L volumetric flask, bottle, and label "Sodium hydroxide 0.1 M" with date of preparation, technician's initials, and an expiration date of 1 year. Store at room temperature.

(3) Add 50 ml of solution 1 and 39.1 ml of solution 2 to a 100-ml volumetric flask and QS to 100 ml with reagent water type I.

(4) Measure the pH and adjust, if necessary to 7.6.

(5) Bottle and label "Phosphate Buffer 0.056M, pH 7.6", with date of preparation, technician's initials, and an expiration date of 1 year. Store at room temperature.

q. Ehrlich's Reagent

(1) Combine in a 250-ml brown glass bottle:

(a) P-dimethylaminobenzaldehyde, Sigma; protect from light	2.0 g
(b) HCl concentration (reagent grade)	25 ml (5 ml repipette)
(c) Glacial acetic acid, (analytical grade, EMS)	75 ml (25-ml repipet)

(2) After dissolution, label as "Ehrlich's Reagent" with the date and time of preparation, technician's initials, and an expiration date and time of 6 hours. Store at room temperature.

r. Ammonium hydroxide, reagent grade ACS, MCB. Store under fume hood.

s. Reagent water type I. Place in bottle with a 5-ml repipet attached.

t. Ion-exchange resin columns (Whale Scientific), for qualitative PBG determination. Store at room temperature. Refer to expiration date on box.

u. Porphobilinogen. Porphyrin Products, Logan, Utah. Store in refrigerator; protect from light.

4. Calibration

a. Porphobilinogen standard, 1,000 ug/ml STOCK:
(1) Porphobilinogen, 100 mg
Porphyrin products, refrigerate, protect from light
Phosphate buffer, pH 7.6, QS to 100 ml.
(2) Dissolve in a 100-ml volumetric flask and freeze in 1-ml aliquots in 10- X 75-mm capped test tubes. The stock is stable at -20 ^{o}C for at least 3 months. A pink color may develop during prolonged storage but the standard is nevertheless acceptable.

b. Porphobilinogen standard, 10 ug/ml working stock:
   (1) Combine in a 50-ml volumetric flask:
       (a) Porphobilinogen 1,000 ul/ml standard, 500 ul
       (b) Reagent water type I, QS to 50 ml
   (2) Use a 500-ul volumetric pipette to dispense the standard. The standard can be used for 2 days if kept refrigerated and protected from light.

c. Working standards are prepared fresh daily and run in duplicate with each assay.

PBG STPS ug/ml	Dilution	Ml of 10 ug/ml PBG	Type I Water	Total Volume
0.5	1:20	0.5	9.5	10.0
1.0	1:10	1.0	9.0	10.0
2.0	1:5	2.0	8.0	10.0
3.0	3:10	3.0	7.0	10.0
4.0	2:5	4.0	6.0	10.0
5.0	1:2	5.0	5.0	10.0

The above standards are prepared from the 10 ug/ml stock by dispensing the 10 ug/ml stock with the Accuprep into 10-ml volumetric flasks. QS to 10 ml with type I water.

5. Quality Control Material

Control material consists of a 12-hr urine specimen obtained from a normal volunteer and spiked with PBG standard. A volume of 1,800 ml of urine is filtered through a Millipore filter to remove bacteria. Add to the filtered urine 4.5 ml of 1,000 ug/ml PBG Standard, mix well, and freeze in 9 ml aliquots at -20 °C. Aliquots are stable at this temperature for 6 months. The control is run in duplicate at the beginning and end of each analytical run.

In addition to running controls, two spiked samples are included in each run. The spiked samples are prepared by placing 3.50 ml of participant urine into each of four labeled test tubes. To two of these tubes add 0.50 ml of 2.0 ug/ml PBG Standard. To the other two tubes add 0.50 of 4.0 ug/ml PBG Standard. The expected results of these spiked samples are 0.25 ug/ml and 0.50 ug/ml.

6. Procedure

a. Allow all reagents, standards, and participant specimens to come to room temperature before testing.

b. Make up standards as described in calibration section.

c. Label two resin columns and two test tubes (16 X 100 mm) for each participant, control, and standard. Snip the tips of the columns with scissors and remove column caps. Place in draining rack with drain reservoir. Be sure the columns are upright and the tips are free. Allow to drain completely without further disturbing the column packing (10 minutes).

d. Insert funnel into each resin column. Using a repipet, deliver 5.0 ml type I water to each column. Allow to drain into waste container.

e. To each of the two labeled test tubes add 4.0 ml of participant sample, control, or standard with an MLA adjustable pipet. To each of these tubes, add 0.4 ml ammonium hydroxide with an Eppendorf pipet set to dispense 0.4 ml. Vortex.

f. Transfer the contents of each tube to its respective column and allow to drain completely into the waste container.

g. With a repipet add 5.0 ml distilled water to each column. Allow to drain completely into waste container.

h. Place columns in labeled 16- X 100-mm test tubes in a rack.

i. Using a repipet, add 4.0 ml 1M acetic acid to each column and collect the eluate. Also add 10 ml acetic acid to a tube labeled "BLANK."

j. Vortex the eluates well and immediately transfer 1 ml of each eluate to duplicate labeled tubes. Prepare eight reagent blanks by pipeting 1 ml of 1M acetic acid into eight labeled tubes. (Avoid picking up resin that may have come from the column.)

k. Using the Eppendorf pipet set to 1 ml, add 1 ml Ehrlich's reagent to each participant, control, and standard tube. Vortex well and allow to stand at room temperature 6-8 minutes. (Add the Ehrlich's reagent at 30-second intervals to allow reading of the reaction tubes at uniform time intervals.)

l. Read and calibrate the Beckman Du-7, using the blanks prepared.

m. Flush the Beckman Du-7 with 2 blanks before and after reading controls to eliminate carry-over.

n. Read and record the absorbance while in the multiwavelength mode of the Du-7 at 535, 555, and 575 against the reagent blank.

7. Calculations

a. To eliminate the effects of interfering chromogens, apply the Allen correction to each participant/control/standard value:

A Corr = 2 A 555 - (A 535 + A 575)

b. Use the linear regression program in the CHC or the IBM PC/XT computer to evaluate the standard curve and obtain participant results (in ug/ml).

c. After this result, the 12-hour urine total volume and the urine creatinine results have been verified, use the force calculations mode to perform the following calculation:

$$\text{PBG (mg/g creat)} = \frac{\text{PBG (in ug/ml)}}{\text{urine creatinine/100 (in mg/dl)}}$$

8. References

a. Labbe, RF. Porphyrins and related compounds. In: Tietz, NW, ed. Fundamentals of clinical chemistry. Philadelphia: WB Saunders, 1976:461-3.

b. Whale Scientific. Urinary porphyrins and porphobilinogen: qualitative screen for urinary porphyrins and porphobilinogen by ion exchange column. Commerce City, Colorado: Whale Scientific, 1979.

V. ANALYTICAL METHODS

H. Quantitative Urinary Porphyrin Screen

1. Principle

Quantitation of excreted porphyrins is a central feature of the diagnosis of various disorders of the porphyrin metabolism. Urinary porphyrins exist as mixtures of two major fractions, uroporphyrin and coproporphyrin.

In this procedure, the second derivative of the absorption spectrum in the region of the Soret-band maximum (wavelength = 400-405 nM) is used to determine to total concentration of porphyrins in urine.

2. Specimen Collection And Storage

a. No participant preparation is necessary.

b. A well-mixed aliquot (5-10 ml) of a 12-hour urine collection is used for this test. Urine specimens are collected in a container with 2 g sodium carbonate. The specimen should be fresh, kept cold, and protected from light.

c. The specimen is stable for 24 hours at 2-8 ^{o}C. For longer storage, freeze at -20 ^{o}C.

3. Reagents, Supplies, And Equipment

a. Glass test tubes, disposable: 16 X 100 mm

b. Spectrophotometer (a Beckman Du-7) capable of plotting second derivative scans

c. Accuprep (Beckman) capable of dispensing 1-1000 ul. Refer to Accuprep manual before using.

d. Class "A" volumetric flasks: 10 ml and 5 ml

e. Coproporphyrin Fluorescence Standard: 0.5 ug per ml. Porphyrin Products, Inc., P.O. Box 31, Logan, Utah. Store at 0-4 ^{o}C. Protect from light.

f. Uroporphyrin I Fluorescence Standard: 0.5 ug per ml. Porphyrin Products, Inc., P.O. Box 31, Logan, Utah. Store at 0-4 ^{o}C. Protect from light

g. H_2O_2: 30% reagent grade

h. Hydrochloric acid: 1.25 mol/L

(1) In a 1-L volumetric flask add 103 ml concentrated HCl to 600-700 ml water (reagent grade ACS, MCB reagent, corrosive). QS to 1 L with reagent grade water. To avoid explosion, add concentrated acid to water and NEVER water to concentrated acid.

(2) Label as "1.25M HCl" with date of preparation, technician's initials, and expiration date of 1 year. Store at room temperature.

4. Calibration

a. Porphyrin Stock Standard (500 ug/L)

(1) Combine 30 ml. Coproporphyrin Fluorescence Stardard (0.5 ug/ml, Porphyrin Products, Inc.) with 10 ml Uroporphyrin Fluorescence Standard (0.5 ug/ml, Porphyrin Products, Inc.).

(2) Label as "Porphyrin Screen Standard 500 ug/L," with preparation date, technician's initials, and expiration date of 3 months. Store at 2-4 °C. Protect from light.

b. Working Standards

(1) A primary working standard (250 ug/L) is made by adding 5 ml of the stock standard (500 ug/L) to a 10-ml volumetric flask; QS with 1.25M. HCL to 10 ml.

(2) The remaining working standards are prepared as shown below.

Porphyrin Standard ug/ml	Dilution	Mls of 250 ug/ml Standard	QS total volume with 1.25M HCL
200	4:5	4	5 ml
100	2:5	2	5 ml
50	1:5	1	5 ml
25	1:10	0.5	5 ml

Standards are run in duplicate with each assay.
Standards are stable for 1 week when stored at 2-4 °C.

5. Quality Control Material

The control material consists of pooled human urine, spiked with porphyrins. The control is assayed in duplicate at the beginning and end of each analytical run.

6. Procedure

a. Porphyrin Screen

(1) Allow standard and participant specimens to come to room temperature before testing.

(2) Label two 16- X 100-mm test tubes for each participant. Place 0.5 mls of sample and control into each labeled tube. Add 2 ml 1.25M. HCL and 0.025 ml H2O2 (30%). Vortex.

(3) Using the Beckman Du-7 spectrophotometer, scan each sample from 430 nm to 380 nm. The operating parameters on the Du-7 are to be set as follows:

(a) Scan (wavelength): (today's date)
(b) Function: (2 Der)
(c) Starting (wavelength): 430
(d) Ending (wavelength): 380
(e) Speed: (120)
(f) Span: 0.002
(g) Fill time: 5.0 seconds
(h) Calibrate with 1.25M HCL

(4) When the Du-7 has finished scanning, change Delta from 2 to 16 using (SEL) button in middle of spectrophotometer. If necessary adjust the high and low range of optical density (OD) to get entire scan on the graph. Record the OD of the lowest point on the scan between 400 and 405 nM. To find lowest point, move curser to left or right, using curser arrows.

7. Calculations

a. Use the linear regression program in the CHC or the IBM PC/XT computer to evaluate the standard curve and obtain the participant results (in ug/L).

b. After this result and the urine creatinine results have been verified, use the force calculations mode to perform the following calculation:

$$\text{Total Porphyrin (in ug/g Creatinine)} = \frac{(\text{Uncorrected Porphyrin (in ug/L)} \times 5) \times (\text{Total Urine Volume (in ml)} \times 1{,}000)}{(\text{Urine Creatinine (in g/12-hour urine)} \times 1{,}000)}$$

8. References

Jones KG, Sweeney GD. Quantitation of urinary porphyrins by use of second-derivative spectroscopy. Clin Chem 1979; 25 (no.1):71-4.

V. ANALYTICAL METHODS

I. Urinary Porphyrins
High Performance Liquid Chromatography (HPLC)

1. Principle

Porphyria is an hereditary or chemically induced disease characterized by excessive porphyrins in body fluids. Type I porphyrins are the principal porphyrin constituents in normal urine. They are unused byproducts of the heme biosynthetic pathway. Type III porphyrins are precursors to heme and are not observed in normal urine. There are five urinary porphyrins. Most normal urine samples contain coproporphyrin and perhaps a small amount of uroporphyrin. The heptacarboxyl-, hexacarboxyl-, and pentacarboxyl-porphyrins are found in urine in trace amounts.

High performance liquid chromatography (HPLC) is used to determine the five principal urinary porphyrins. The method involves a 15-minute gradient separation on a Bondapak phenyl column. Before the sample can be analyzed, the urine sample must be adjusted into the acidic range and oxidized.

2. Specimen Collection And Storage

a. No participant preparation is necessary

b. The 12-hr urine specimen is collected in a container with 2 g of sodium carbonate and is kept cold and protected from light during collection. Before the mixture is analyzed for porphyrius by this procedure, 0.05 g of EDTA is added to a 50-ml aliquot.

c. The specimen is stable for 24 hours at 2-8 °C. For longer storage, store specimen at -20 °C.

3. Reagents, Supplies, And Equipment

a. Glass test tubes, disposable 10 X 75 and 16 X 100 mm.

b. Pipets: Glass 5 3/4-inch Pasteur pipets
MLA with disposable tips, 0.2, 0.1, 0.02, and 1.0 ml.
Serological pipets 1 and 2 ml; volumetric pipets, 1 ml.

c. Hamilton 100-ul syringe

d. Sample cups, 0.5 ml

e. Erlenmeyer flasks 4,000 ml

f. Analytical balance Mettler AE166

g. pH meter and standards

h. pH paper, 3-5 range

i. IBM 9533 ternary gradient liquid chromatograph

j. Perkin Elmer LS4 spectrofluorometric detector

k. IBM PC/XT personal computer

l. Nelson Analytical 760 series interface

m. Epson printer

n. Standard Diluent

(1) 180 ml HPLC grade methanol, Fisher
(2) 120 ml reagent grade type 1 water
(3) 1.14 g EDTA, Sigma
(4) After dilution, label as "Standard Diluent" with date of preparation, technician's initials, and expiration date of 6 months. Store tightly capped at room temperature.

o. 1-pentanesufonate, sodium salt solution, Fisher

(1) 12 g 1-pentanesulfonate, sodium salt, HPLC grade
(2) 0.151 g EDTA
(3) 3,900 ml reagent grade type 1 water
pH to 2.1 with 1N H_2SO_4.
QS to 4,000 ml.
(4) After dilution, label as "1-Pentane/Sulfonate Sodium Salt" with date of preparation, technician's initials, and expiration date of 6 months.

p. 61% Methanol-Solvent A

(1) 610 ml HPLC grade methanol
(2) 390 ml sodium 1-pentanesulfonate solution
(3) In a 1-L graduated cylinder, add the required amount of methanol. Add 390 ml of Na pentane/sulfonate. Let this mixture sit in the graduated cylinder 5-10 minutes, or until all bubbling ceases. QS to 1 L with Na pentanesulfonate. Make a total of four 1-L liter batches at a time, bottle, and label "Solvent A" with preparation date, technician's initials, and an expiration date of 6 months. Store at room temperature.

q. 74% Methanol-Solvent B

(1) 740 ml HPLC grade methanol
(2) 260 ml 1-pentanesulfonate sodium salt solution
(3) In a 1-L graduated cylinder, add the required amount of methanol. Add 260 ml of Na pentane/sulfonate. Let this mixture sit in the graduated cylinder 5-10 minutes, or until

all bubbling ceases. QS to 1 L with Na pentanesulfonate. Make a total of four 1-L batches at a time, bottle, and label "Solvent B" with preparation date, technician's initials, and an expiration date of 6 months. Store at room temperature.

r. 80% Methanol-solvent C (wash solution)

(1) 800 ml HPLC grade methanol
(2) 200 ml reagent grade type 1 water
(3) Make 4 L, bottle, and label "Solvent C" with preparation date, technician's initials, and an expiration date of 6 months. Store at room temperature.

s. Waters Bondapak phenyl column

t. IBM Octadecyl mini-analytical column.

u. Porphyrin Acid Chromatographic Marker Kit, CMKA, Porphyrin Products, Logan, Utah

4. Calibration

a. Stock standard

(1) Add 1 ml of standard diluent to a vial of porphyrin standard, using a volumetric pipet. Vortex and label with date of reconstitution. Stable 1 week.
(2) Wrap vial in foil.
(3) Standard must sit overnight at room temperature before being used.

b. Working standards

(1) Standard A: 1.9 ml standard diluent + 0.1 ml stock
(2) Standard B: 1.2 ml standard diluent + 0.4 ml standard A
(3) Standard C: 0.6 ml standard diluent + 0.6 ml standard B
(4) Standard D: 0.6 ml standard diluent + 0.6 ml standard C

c. Porphyrins standard concentrations in ug/L:

	URO	HEP	HEX	PENT	COPRO
A	377	357	337	317	297
B	95	89	85	79	75
C	47	45	42	40	37
D	24	23	21	20	18
E	0	0	0	0	0

5. Quality Control Material

The control material consists of lyophilized urine control BioRad Lyphochek level 2. The control is assayed after the standards and two thirds of the way through each analytical run.

The control material is reconstituted with 25 ml type I reagent grade water. 0.025 g EDTA and 0.030 g Na_2CO_3 are added. The material is then frozen in 0.2-ml aliquots in foil-covered containers. It is stable at -20 °C for 6 months.

As a further check on assay performance, porphyrin standard B or C is run at the end of the participant samples to verify that all standard peaks are occurring within their specified windows.

If for any reason during the run retention time seems to have shifted, a standard should be run, and, if necessary, restandardization should be performed.

6. Procedure

a. Label one 10- X 75-mm test tube for each standard. Make standards according to directions in calibration section.

b. Fill reservoir bottles with the appropriate solvent.

c. Open valve to vacuum pump and apply vacuum for 15 minutes.

d. Close valve to vacuum pump; turn off pump.

e. Open helium valve and turn on helium tank with 3-5 psi on the gauge.

f. Open the pump purge valve and leave it open.

g. Bleed lines by programming.

(1) (Pump) (Solvent A) (100) enter
(2) (Flow) (5.0) enter, let run 2-3 minutes.
(3) (Pump) (Solvent B) enter, let run 2-3 minutes.
(4) (Pump) (Solvent C) enter, let run 2-3 minutes.
(5) (STOP)

h. While purge valve is still open, start operating procedure /Proc/ /0/ /Start/.

i. Close purge valve. Let solvent system run through at least one gradient before injecting a sample.

j. Turn on fluorescence detector, IBM computer and printer, and the Nelson interface box.

k. The LC Wavelength Program must be entered daily. This changes the emission and excitation wavelengths to the optimum conditions for each peak. The emission and excitation wavelengths are established by experimentation. The times are those times in between peaks. The detector will automatically

return to zero after each change. The wavelengths and times are as follows (times may vary with columns and solvent conditions):

Time	Excitation (Wavelength)	Emission (Wavelength)
0.0	408	640
6.5	407	620
8.9	406	615
10.5	405	600
12.0	405	600

(1) Press LC program button so that the red light above the key is lit.
(2) Empty the file block by holding down /CE/ key and pressing the /LC prog/ key.
(3) Press hit the file key. An E will be written in each location.
(4) Key in a time and press the /Time/ key.
(5) Key in the corresponding excitation wavelength and press /EX/ key.
(6) Key in the corresponding emission wavelength and press /EM/ key.
(7) Press the /File/ key before 10 seconds.
(8) Key in next Time, EX, EM, File.
(9) For the last entry press /CE/. A C will be written in each location.
(10) Type in 15.0 and press /Time/ /File/ /File/.
(11) To view program, press FILE key. Each time key is pressed a step will be displayed.

l. At the C> on the CRT screen, type CD/HPLC.

m. At the next C> type MENU.

n. Each set of participant information must be downloaded to the interface. This can be done in one of two ways:

Note: Standards are run by using method A. The rest of the samples are run by using method B.

(1) Enter "acquire data mode" of the main menu (option 0).

(a) :Press the F2 function key. Answer the following questions:

Method = /Porphyri/
Raw Data File / / i.e., A, B, C, D.
Sample Name / / i.e., Std A, Std B.
Leave the answers to all the other questions as they are.

(2) Make a sequence chart. After the first download, the computer will automatically download the participant data in the chart.

(a) Parameter 3. Generate or modify a sequence.
(b) Edit an existing file (n).
(c) Display directory (n).
(d) Answer the following questions for each sample:

Sample name / / i.e., sample number - participant number
Method /Porphyri/
Raw data file name / / i.e., Mon, Tues, etc.
Amount injected 1.0
Int. std. amt. 1.0
Dilution factor /1.2/

(e) Place the control after the standard and approximately two-thirds through the run.

(f) Store the chart (F9). Print the chart (F8).

(g) The chart can be edited throughout the day for cancellations, add ons, etc. Follow the computer prompts.

(h) To download the sequence chart to the interface, enter acquire data mode of the main menu (option 0).

(i) Press the F2 function key and answer the following questions:

Method /porphyri/
Raw data file name / /, i.e., Mon, Tues, etc.
Use sequence table /y/
Name of sequence table / /, i.e., Mon, Tues, etc.
Sample name / /

(j) Each subsequent participant sample will appear on the screen. An injection can be made without any further adjustments to the computer.

o. To process a sample (standards, controls, and participants):

(1) Participant samples must be oxidized with H_2O_2 before assaying. Place 1 ml of a well-mixed urine sample in a labeled 10- X 75-mm tube. Add 0.1 ml of 10% H_2O_2. Incubate for 30 minutes before assaying.
(2) Take a 200-ul aliquot of the oxidized sample and place it in a 0.5-ml sample cup.
(3) Add 20 ul of an appropriate dilution of H_2SO_4 to adjust the pH to 3.0, using pH paper.
(4) Rinse the 100-ul syringe with an aliquot of the acidified sample and discard.
(5) Draw up 100 ul of sample, making sure that there are no air bubbles.
(6) Download the participant data to the interface (see step 14).

(7) When the HPLC system is ready for a sample (the timer on the IBM control box should be at 16 minutes or longer so that the original solvent conditions are present), turn the rheodyne valve to the load position.
(8) Insert the needle into the port.
(9) Inject.
(10) Turn the valve to the inject position and simultaneously press the START button twice (on the IBM control box). This starts the interface and detector timers and resets the IBM control box timer to 0.

p. After the standard curve has been run, the areas must be entered into the computer:

(1) Parameter 1 of the main menu--Generate or modify a method.
(2) Load an existing file /yes/ /porphyri/.
(3) Option 4 - Edit component names.
(4) Option 0 - Edit all.

Scroll through the first screen and enter areas in the appropriate locations. Change the retention times relative to the standards for each component, if necessary.

(5) Store method-F9; answer /y/ to overwrite the file.

(6) Return to the main menu--F10.

(7) Record areas and times of standards on the standard log.

q. To shut down:

(1) Let computer finish printing the last sample.
(2) Type /prog/ /0/ /stop/
(3) Change solvents if it is a workday (Mon-Thurs). Apply vacuum and purge.
(4) Type /prog/ /9/ /start/; let the analyzer pump a minute and then close the purge. Program 9 trickles solvent A at 0.2 ml/min all night after the 1-hour wash cycle.
(5) If it is a weekend, be sure the supply of solvent C is sufficient. After stopping program 0, start program 8. This washes with 80% methanol for 1 hour, then shuts the pump off.
(6) Turn off helium tank.

r. Record standards' and controls' areas on QC charts.

s. Keep track of daily source lamp usage.

t. Keep track of the total number of injections on the column.

u. Change guard column when the pressure reaches approximately 250 Barr or if the control does not work.

7. Calculations

   a. After the Ektachem has reported values for urine creatinines, obtain a printout of the worksheet from the computer room.

   b. Use the urine creatinine value mg/dl to calculate the mcg/g creatinine value.

      xxx.x ug/L Porphyrin Component X 100 = xxxx mcg/g creatinine
      xxx.x mg/dl Creatinine

8. References

   a. Hill, RH. Development and utilization of a procedure for measuring urinary porphyrins by high performance liquid chromatography. J Chromatog 1982;232:251-60.

   b. IBM Instruments. IBM LC/9533 HPLC system user's manual. Danbury, Connecticut: IBM Instruments, 1983.

   c. Perkin-Elmer. Model LS-4 fluorescence spectrometer operator's manual. Beaconsfield, Buckinghamshire, England: Perkin-Elmer, 1981.

   d. Porphyrin Products. Porphyrin ester chromatographic marker kit, package insert. Logan, Utah: Porphyrin Products, 1985.

V. ANALYTICAL METHODS

J. Routine Chemical Analyses of Serum and Creatinine in Urine
The Kodak Ektachem 700

1. Overview

Chemical assays of serum and of urine for creatinine are performed by using the Kodak Ektachem 700 autoanalyzer. General procedures for the daily operation of equipment, calibration, and preparation of quality control material are presented and the methods and materials specific to each assay are then described.

2. Kodak Ektachem Procedure

The purpose of this procedure is to assist the technologist in the daily operation of the Ektachem.

a. Check slide supplies. Touch CARTRIDGE LOADING TARGET. Touch DISPLAY INVENTORY. This will give you the total number of slides in the analyzer for each test.

b. Take any reagent cartridges out of the refrigerator or freezer that will be needed. Cartridges removed from the refrigerator must sit at room temperature, in the foil wrapper, for 30 minutes before being placed in the analyzer. Those removed from the freezer must sit for 60 minutes.

c. Perform daily maintenance and record.

(1) Empty slide tip disposal box.
(2) Empty slide disposal boxes.
(3) Remove empty cartridges from slide supplies.
(4) Take ambient temperature and record in maintenance log. Touch DIAGNOSTICS. Touch ENVIRONMENTAL MONITORING. Record. Repeat in the afternoon.

d. Load slide cartridges into slide supply, using the CARTRIDGE LOADING Target.

(1) Touch LOCATE MODE target. The analyzer will present the nests. Touch any nests that have 00 slides. The analyzer will bring these nests to the slide supply door.

Remove the empty cartridge. Replace it with a new cartridge. Touch CHANGE SUPPLY and repeat as necessary for slide supply 2.

(2) Touch NORMAL MODE. The analyzer will bring an empty nest position to the slide door. Add a new cartridge and close the door. Repeat until all cartridges are loaded. Touch

CHANGE SUPPLY and repeat for slide supply 2 until all cartridges are loaded.

Note: Make sure that you are loading the same lot number. Mark the load date in pencil on the side of the cartridge. Most cartridges are stable for 1 week with the exception of creatinine and ammonia, which are stable for 48 hours. Those stable for 2 weeks are ALB, BUN, AST, ALT, LDH, GGT, ALKP, CHOL, GLU, TBIL, and TRIG. Slide supply 2 contains the enzyme, glucose, creatinine, and ammonia cartridges. All other cartridges belong in slide supply 1.

e. When you have finished loading cartridges, touch RETURN. The analyzer will automatically update the contents and return to the main menu.

f. The analyzer is now ready for sampling participant specimens or for a calibration run.

(1) Touch SAMPLE PROGRAMMING target on the CRT screen.

(2) Touch TRAY NAME. Type in a tray name and press ENTER on the keyboard.

(3) Touch a quadrant target. The tray name will appear in that quadrant. Cups 1-10 will appear to the right of the screen.

(4) Touch CUP 1. A new screen will appear.

(5) Type in sample ID number in sample ID target and press ENTER on the keyboard.

(6) Touch the desired test or panel (all). This panel has been preprogrammed for the tests that we are running. (See Ektachem procedure manual to change a panel.)

(7) Press ENTER PROGRAM target.

(8) Screen will reappear with cup 2 ready to be programmed. Continue through cup 10.

(9) To program a new tray, repeat steps b-g.

(10) Turn sampling ON.

3. Daily Run

a. Pull out:

(1) Frozen aliquots of Kodak I and II controls.
(2) Aliquots of the creatinine control.
(3) Calibrator 1 (reconstitute after it has thawed).

b. Check cartridge loading for slides needed; keep at least 100 in per day--2 cartridges of 50, 200 of creat, NH3, Chol--4 cartridges of 50.

c. Do daily maintenance. Take analyzer ambient temperature under DIAGNOSTICS and ENVIRONMENTAL MONITORING. Record.

d. Label tubes for urine creatinine and HDL cholesterol. Program trays without assigning the quadrant.

e. Do urine creatinines 10 at a time; i.e., in 3 groups of 10.

f. Run Kodak controls and calibrator 1. Record Kodak controls and calibrator 1 results in log. Notify QC supervisor if calibrator 1 values are not within limits.

g. Start running chemistry profiles - 1 tray of 10 at a time. Place participant samples in duplicate, contiguous cups. Program one with the number and one with the number - A; e.g., 8,000 and 8,000-A: Place a Kodak control at the beginning of each tray. Alternate levels, i.e., Kodak I and tray A, Kodak II in tray B, Kodak I in tray C, etc.

h. Upon receiving all the participant specimens, perform the HDL procedure, and run the supernatants on the Ektachem.

i. Continue to run profiles.

j. Take ambient temperature again and record in maintenance log.

k. Enter and verify results.

l. Clean up. Check cartridge loading for the next day.

4. Reagents

a. Storage:

(1) Cartridges containing Kodak Ektachem clinical chemistry slides must be stored under conditions that will protect the slides from corrosive elements, high temperatures, and high humidity. For assays ALKP, CK, ALT, AST, LDH, and GGT, unopened slide cartridges should be stored in a freezer at or below -18 °C (0 °F). Product dating is predicted on freezer storage; however, unopened cartridges may be stored at 2-8 °C for a maximum of 4 weeks. For ALB, CHOL, TBIL, TP, TRIG, SCREA, UCREA, BUN, GLU, HDLC, BU, and BC, slide cartridges should be stored in the refrigerator at 2-8 °C or at lower temperatures. Cartridges also may be stored in a freezer, but they will require a longer warm-up period.

(2) Slides may be left in the analyzer's slide supply for up to 2 weeks. If the slides have not been exhausted within 2 weeks, they should be replaced with fresh slides.

(3) Slide cartridges should be removed from the analyzer's slide supplies before the analyzer is shut down. When they have been removed, immediately place partially used cartridges in a dry, airtight container, and refrigerate at 2-8 °C. A round, widemouth, glass bottle with a tight-sealing screw cap is a suitable container for 2 weeks of refrigerated storage. The bottle should be of minimum size to provide the least air space. A small amount of anhydrous calcium sulfate desiccant, such as Drierite (W. A. Hammond Drierite Co.), should be placed in the bottle to protect the slides from moisture. Use tissue or another suitable barrier to prevent the desiccant from contaminating the slides. Bottles and desiccant are available from most laboratory supply dealers.

(4) Slides should be used before the date that appears on the cartridge box.

b. Preparation:

(1) Slide cartridges must be removed from the freezer (or refrigerator) and allowed to reach room temperature before they are loaded into the analyzer. Cartridges stored under freezer conditions require a minimum of 1 hour to reach room temperature. Refrigerated cartridges require at least 30 minutes.

(2) To prevent condensation on the slides, remove the cartridge from its wrapper or other airtight container just before inserting it into the analyzer's slide supply and only after it has reached room temperature. Unopened cartridges left at room temperature should be loaded into the analyzer within 24 hours.

5. Calibration

To be done monthly:

a. Remove the calibrators from the refrigerator (calibrators 2, 3, 4, 6) and freezer (calibrators 1, 5, 7) and allow them to come to room temperature. Be sure that the diluent bottles for calibrators 1, 2, and 5 also come to room temperature.

(1) Reconstitute calibrators 3, 4, and 6 with 3.0 ml of deionized water, using a class A volumetric pipet. Reconstitute calibrators 1, 2, 5, and 7 with 3.0 ml of their respective diluents.

(2) Allow the calibrators to stand for 30 minutes; gently swirl the bottles periodically. (DO NOT SHAKE.)

(3) Place the reconstituted calibrators in the refrigerator for an additional 15 minutes to allow the enzyme components to reactivate fully.

(4) Warm to room temperature for a minimum of 5 minutes before calibrating.

(5) Once reconstituted, calibrators are stable for 24 hours. Refrigerate to maintain stability. (Creatinines and enzymes should be calibrated within 1 hour.)

b. Calibration Procedure:

(1) Perform the White reference test (see Procedure 16.4.3, pp. 16-23 Ektachem Manual).

(2) Touch CALIBRATION PROGRAMMING.

(3) Type the name of the desired group. If the group does not exist, type Y and press ENTER.

(4) Touch the test targets to program the test(s) to be calibrated in this group.

Note: The following groups have been established to facilitate monthly calibration of all analytes:

Group 1: Glucose, BUN, total protein, total bilirubin, BU, and BC (use bottles 1, 2, 3, 4, and 6).

Group 2: AST, ALT, LDH, CH, ALKP, GGT, albumin (use bottles 1, 3, 5, and 7).

Creatinine: Use bottles 1, 3, 5, and 7.

(5) When you have finished selecting the test, touch ASSIGN BOTTLE LOT NUMBERS. This screen displays calibrator lot numbers required to calibrate the tests in the calibration group. (The last digit of the lot number is the calibrator bottle number.) If lots are acceptable, touch RETURN

(6) Follow steps c-e to enter groups 2 and 3.

(7) Once the groups are programmed, type the name of the group and enter. That particular group name should now be flashing on the screen. Touch LOAD GROUP. This screen will display the group name and assign a quadrant. It will also display which calibrators go into which cups.

(8) Touch GROUP IS LOADED target and repeat steps g-h for other groups.

(9) Turn sampling ON. The analyzer should begin sampling at the first cup of the nearest quadrant.

(10) Record all values in the calibration book. Also record QC values in duplicate in the QC notebook.

(11) Run controls and validate creatinine by running calibrator 1 as a participant sample. This value should be ±1 SD of SAV.

Note: Recalibration is required 1) at a change in slide lot number or generation number, 2) when quality control limits are exceeded; 3) when service OTHER THAN routine maintenance has been performed, 4) following entry of a new White reference correction factor for a reflectometer, and 5) when the laboratory's ambient temperature has changed ±5 °C or more from the temperature at the time the test was calibrated.

(12) To perform calibrations, the following calibration models are employed for the given types of assays:

(a) Multiple-point rate: ALKP, CK, ALT, AST, LDH, and GGT

(b) End-point colorimetric: ALB, CHOL, TP, TRIG, BUN, GLU, HDL, and BU/BC

(c) Blank-corrected (dual wavelength) colorimetric: TBIL, SCREA, and UCREA

(13) To check a calibration curve for a specific test, run those calibrators that contain the analyte. Compare the value to the SAVs.

(a) Cal 1: All except HDLC
(b) Cal 2: GLU, BUN, SCREA, UCREA, NH_3, CHOL, TRIG, and TP
(c) Cal 3: GLU, BUN, SCREA, UCREA, NH_3, ALB, HDLC, BU, and BC
(d) Cal 4: TRIG, TP, TBIL, BU, and BC
(e) Cal 5: AST, ALT, LDH, CK, ALKP, GGT, HDLC, SCREA, UCREA, NH_3, and ALB
(f) Cal 6: CHOL, TBIL, and BU
(g) Cal 7: AST, ALT, LDH, CK, ALKP, GGT, and HDLC

6. Quality Control Material

a. Chemistry Profiles Controls

(1) Kodak I and II controls are to be run daily. Remove vials and their respective diluents from the freezer and allow

them to come to room temperature. Reconstitute, using a 5.0-ml volumetric pipet. When completely reconstituted, IMMEDIATELY aliquot into 0.5-ml tubes and freeze. Assay each level in quadruplicate, interspersed throughout the run. Record in the QC notebook. Aliquots are stable in the freezer for 5 days.

(2) Kodak Ektachem calibrator 1 will be run daily in duplicate. Remove vial and its diluent from the freezer and allow them to come to room temperature. Reconstitute, using a 3.0-ml volumetric pipet. Let stand for 30 minutes. Swirl occasionally. Place in refrigerator for 15 minutes. Remove and let vial warm for 5 minutes before assay. Assay in duplicate. Record in QC notebook. If value is out of range, make up a new bottle. If it is still out of range, recalibrate.

b. HDL Cholesterol Controls:

(1) Sigma HDL LO and HI controls are used. The manufacturer is Sigma Diagnostics, P.O. Box 14508, St. Louis, MO 63178. Store dry vials in the refrigerator at 2-6 °C until expiration date on the label. After having been reconstituted, controls are stable for at least 5 days when stored at 2-6 °C.

(2) To reconstitute, add 3.0 ml of ACA water to vial, using a 3-ml volumetric pipet. Allow to stand at room temperature for 10 minutes. Swirl gently and mix before each use. Treat control serum in the same manner as the test samples. Run each control level in quadruplicate interspersed throughout the run.

c. Urine Creatine Controls:

(1) BioRad Lyphochek levels I and II urine controls are to be run. Unreconstituted material should be stored at 2-8 °C and is stable until the expiration date on the label.

(2) To reconstitute: Add 25 ml of distilled water, using a 25-ml volumetric pipet. Allow the material to sit for 5-10 minutes. Swirl contents until homogeneous. Freeze 1-ml aliquots.

(3) To use: Treat the control with the ion exchange resin, just as you would the participant samples. Run controls in quadruplicate, one each at the beginning and end of the run with two additional replicates inserted randomly within the run.

7. ALANINE AMINOTRANSFERASE (SGPT) (ALT)

a. Test Principle: The assay is based on alanine aminotrans-ferase (ALT) catalyzing the reaction of L-alanine and alpha-ketoglutarate in the presence of pyridoxal-5-phosphate (PLP) to generate glutamate and pyruvate. The pyruvate is then converted to lactate by lactate dehydrogenase in the presence of NADH, which is oxidized at 37 °C to NAD+. The oxidation of NADH is monitored by reflectance spectrometry at 340 nm and is used as a measure of ALT activity.

b. Specimen Collection and Preparation: Analysis should be performed on a serum or plasma specimen that has been collected by standard venipuncture technique. For plasma specimens, heparin may be used as an anticoagulant.

Special Precautions: Avoid freezing the sample, since freezing and thawing have been reported to cause large losses in ALT activity. Sample size is 11 ul.

c. Test Reagents:

Ingredients: Reactive ingredients are lactate dehydrogenase; L-alanine; sodium alpha-ketoglutarate; nicotinamide adenine dinucleotide, reduced; and sodium pyridoxal-5-phosphate. Other ingredients include pigment, binders, buffer, surfactants, polymer cross-linking agents, and preservative.

Note: The dynamic range of the ALT assay is directly proportional to the amount of NADH available on the slide. Since NADH is sensitive to temperature, humidity, and light, any excessive or prolonged environmental exposure could result in an elevated sample (yet still within the dynamic range) being flagged by the analyzer as requiring dilution. Therefore, slide storage and handling conditions must be adhered to.

d. Reference: Eastman Kodak. Alanine aminotransferase, Ektachem test methodology. Rochester, New York: Eastman Kodak, 1984.

8. ALBUMIN (ALB)

a. Test Principle: The analysis is based on the binding of bromcresol green dye to albumin, resulting in a substantial shift in the wavelength of light absorbed by the free dye. The density of the albumin-bound dye is related to the concentration of albumin in the sample and is measured spectrophotometrically at 620 nm.

b. Specimen Collection and Procedure: Analysis should be performed on a serum specimen that has been collected by

standard venipuncture technique. Participants should be in a fasting state; the tourniquet should be applied so that the pressure it produces is minimal. For plasma specimens, lithium heparin may be used as an anticoagulant.

Special Precautions: No special precautions are required other than removing serum promptly from the clot.

No additives or preservatives are required.

If the specimen cannot be analyzed immediately, it should be refrigerated at 2-8 °C. Specimens should be frozen for long-term storage or shipment. The sample size is 10 ul.

c. Test Reagents: Bromcresol green dye, sodium salt.

d. Reference: Eastman Kodak. Albumin, Ektachem test methodology. Rochester, New York: Eastman Kodak, 1983.

9. ALKALINE PHOSPHATASE (ALKP)

a. Test Principle:

Kodak Ektachem clinical chemistry slides (ALKP) contain a dry, multilayered analytical element coated on a polyester film support. The assay is based on alkaline phosphatase (ALKP) catalyzing the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol at alkaline pH in the presence of buffer and 2-amino-2-methyl-1-propanol. The rate of release of p-nitrophenol at 37 °C, which is monitored by reflectance spectrophotometry at 400 nm, is used as a measure of ALKP activity.

b. Specimen Collection and Preparation: Analysis should be performed on a serum or plasma specimen that has been collected by standard venipuncture technique. For plasma specimens, heparin may be used as an anticoagulant.

Special Precautions: Serum should be removed promptly from the clot. Hemolyzed specimens should not be used. Incompletely clotted samples containing fibrin materials can cause problems in the analyzer metering. The sample size is 11 ul.

c. Test Reagents:

Ingredients: Reactive ingredients are p-nitrophenyl phosphate, 2-amino-2-methyl-1-propanol, and magnesium sulfate. Other ingredients are pigment, binders, buffers, surfactants, polymer crosslinking agent, and preservative.

d. References: Eastman Kodak. Alkaline phosphatase, Ektachem test methodology. Rochester, New York: Eastman Kodak, 1984.

10. ASPARTATE AMINOTRANSFERASE (SGOT) (AST)

a. Test Principle: In the assay for aspartate aminotransferase (AST), the amino group of L-aspartate is transferred to a alpha-ketoglutarate in the presence of sodium pyridoxal-t-phosphate (PLP) to produce glutamate and oxaloacetate. The oxaloacetate formed in the deamination of the L-aspartate is converted to malate by malate dehydrogenase in the presence of NADH, which is oxidized at 37 °C to NAD+. The oxidation of NADH is monitored by reflectance spectrometry at 340 nm and is used as a measure of lactate dehydrogenase activity.

b. Specimen Collection and Preparation: Analysis should be performed on a serum or plasma specimen free from hemolysis that has been collected by standard venipuncture technique. For plasma specimens, heparin may be used as an anticoagulant.

Special Precautions: Serum should be removed promptly from the clot. Hemolyzed specimens should not be used, since red cells will contaminate AST. Incompletely clotted samples containing fibrin materials can cause problems in the analyzer's metering. The sample size is 11 ul.

c. Test Reagents:

Ingredients: Reactive ingredients are lactate dehydrogenase; L-alanine; sodium alpha-ketoglutarate; nicotinamide adenine dinucleotide, reduced; and sodium pyridoxal-t-phosphate. Other ingredients are pigment, binders, buffers, surfactants, polymer cross-linking agents, and preservative.

Note: The dynamic range of the AST assay is directly proportional to the amount of NADH available on the slide. Since NADH is sensitive to temperature, humidity, and light, any excessive or prolonged environmental exposure could result in an elevated sample (yet still within the dynamic range) being flagged by the analyzer as requiring dilution. Therefore, slide storage and handling conditions must be adhered to.

d. Reference: Eastman Kodak. Aspartate ammotransferase, Ektachem test methodology. Rochester, New York: Eastman Kodak, 1984.

11. TOTAL BILIRUBIN (TBIL)

a. Test Principle: Kodak Ektachem clinical chemistry slides (TBIL) are used to measure total bilirubin in serum specimens from patients more than 14 days old.

The method is based on the use of dyphylline and surfactant to dissociate unconjugated and conjugated bilirubin from albumin. These bilirubin fractions, with the albumin-linked bilirubin B, subsequently react with the diazonium salt [4-(N-carboxy-methylsulfamyl) benzene diazonium hexafluorophosphate] to produce azobilirubin chromophores, which have similar molar absorptivities and absorption maxima of about 520 nm.

b. Specimen Collection and Preparation: Analysis should be performed on a serum or plasma specimen collected by standard blood collection technique and removed promptly from the clot. Heparin may be used as an anticoagulant for plasma specimens. Incompletely clotted specimens containing fibrin materials can cause problems in the analyzer's metering.

Special Precautions. Specimens should be protected from light and should be analyzed as soon as possible after they have clotted. Direct exposure to sunlight is reported to cause as much as a 50% loss of serum bilirubin in 1 hour, especially when serum is kept in capillary tubes. Exposure to normal laboratory light can result in significant loss of serum bilirubin after 2 to 3 hours. Loss due to light exposure is reported to be greater when a spectral assay is used. The sample size is 10 ul.

c. Test Reagents:

Ingredients: Dyphylline; [4-(N-carboxymethylsulfamyl) benzene diazonium hexafluorophosphate]; copoly (styrene-co-N-vinylbenzyl quarternary salt-co-divinylbenzene). Other ingredients include pigment, binders, buffer, surfactants, polymer cross-linking agent, and a preservative.

d. Reference: Eastman Kodak. Total bilirubin, Ektachem test methodology. Rochester, New York: Eastman Kodak, 1985.

12. UNCONJUGATED/CONJUGATED BILIRUBIN (BU/BC)

a. Test Principle: Kodak Ektachem clinical chemistry slides (BUBC) are used to determine unconjugated bilirubin (BU) and mono- and di-sugar conjugated bilirubin (BC) in human serum. The other major fraction of bilirubin known to exist in human serum, called delta bilirubin (B), is an albumin-linked bilirubin that is not measured by the BUBC slides. When used with a total bilirubin assay using Kodak Ektachem clinical chemistry slides (TBIL), the concentration of B can be calculated by difference between TBIL and (BC + BU).

BU is the fraction of bilirubin commonly referred to as "indirect-reacting" bilirubin. BC is the fraction of bilirubin commonly referred to as "direct-reacting" bilirubin.

b. Specimen Collection and Preparation: Analysis should be performed on a serum or plasma specimen collected by standard blood collection technique and removed promptly from the clot. For plasma specimens, heparin may be used as an anticoagulant.

Special Precautions: Specimens should be protected from light and should be analyzed as soon as possible after they have clotted. Direct exposure to sunlight is reported to cause as much as a 50% loss of serum bilirubin in 1 hour, especially when serum is kept in capillary tubes. Exposure to normal laboratory light can result in significant loss of serum bilirubin after 2 to 3 hours. However, loss due to light exposure is reported to be less with a spectrophotometric procedure than with a diazo bilirubin assay.

No additives or preservatives are required.

Recommended Storage, Handling, and Shipping Instructions. To keep specimens from becoming contaminated or evaporating, handle them in stoppered containers according to standard laboratory procedures. In addition, direct exposure to light must be avoided. If a specimen cannot be analyzed immediately, the serum should be frozen to keep it from converting from BC to BU. The sample size is 10 ul.

c. Test Reagents: Ingredients: Caffeine (source: coffee bean), sodium benzoate, copoly (styrene-co-N-vinylbenzyl quaternary salt-co-divinylbenzene). Other ingredients are pigment, binders, buffer, surfactants, and a polymer cross-linking agent.

d. References: Eastman Kodak. Unconjugated bililrubin/conjugated bilirubin, Ektachem test methodology. Rochester, New York: Eastman Kodak, 1985.

13. BLOOD UREA NITROGEN (BUN)

a. Test Principle: The analysis is based on a urease-catalyzed hydrolysis of urea to ammonia and carbon dioxide.

Enzymatic determination of urea nitrogen has advantages of specificity and speed and avoids the use of hazardous or corrosive chemicals. After release by the enzyme, ammonia is determined colorimetrically by reacting with an indicator.

b. Specimen Collection and Preparation: Analysis should be performed on serum specimens collected by standard venipuncture technique. No special patient preparation is necessary. For plasma specimens, lithium heparin may be used as an anticoagulant. Plasma collected with sodium fluoride preservative cannot be used.

Special Precautions: Serum should be removed promptly from the clot. If analysis cannot be performed immediately, store specimens as recommended below to avoid loss of urea nitrogen or an increase in ammonia levels due to bacterial action.

If analysis cannot be performed immediately, the serum should be refrigerated at 4 °C for short-term storage (up to 1 week). Serum should be frozen for shipment. The sample size is 10 ul.

c. Test Reagents:

Ingredients: Urease, N-propyl-4 (2,6-dinitro-4-chlorobenzyl)-quinolinium ethane sulfonate.

d. Reference: Eastman Kodak. Blood urea nitrogen, Ektachem test methodology. Rochester, New York: Eastman Kodak, 1985.

14. TOTAL CHOLESTEROL (CHOL)

a. Test Principle: The analysis is based on an enzymatic method. After the cholesterol esters have been hydrolyzed to cholesterol, hydrogen peroxide is generated from the catalyzed oxidation of total cholesterol. The peroxide then oxidizes a leuco dye in the presence of peroxidase to produce a dye having a maximum absorption at about 650 nm.

b. Specimen Collection and Preparation: Analysis should be performed on a serum or plasma specimen that has been collected by standard venipuncture technique. For plasma specimens, lithium heparin may be used as an anticoagulant.

Special Precautions: Promptly remove the serum from the clot.

If the specimen cannot be analyzed immediately, refrigerate it at 2-8 °C. Specimens should be frozen for long-term storage or shipment. The sample size is 10 ul.

c. Test Reagents:

Ingredients: Cholesterol oxidase; cholesterol ester hydrolase; peroxidase; 2-(3,5-dimethoxy-4-hydroxy-phenyl)-4,5-bis (4-dimethylaminophenyl) imidazole.

d. Reference: Eastman Kodak. Cholesterol, Ektachem test methodology. Rochester, New York: Eastman Kodak, 1985.

15. HDL CHOLESTEROL (HDLC)

a. Test Principle: The analysis of cholesterol in high-density lipoproteins (HDL) from serum or EDTA-treated plasma specimens

is performed on Kodak Ektachem analyzers with Kodak Ektachem clinical chemistry slides (CHOL). After low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) have been removed by a well-established procedure using dextran sulfate, the HDL sample is analyzed by the Ektachem slide for cholesterol.

The slide consists of a dry, multilayered analytical element coated on a polyester film support. The analysis is based on an enzymatic method similar to that proposed by Allain et al. After the cholesterol esters have been hydrolyzed to cholesterol, hydrogen peroxide is generated from the catalyzed oxidation of total cholesterol. The peroxide then oxidizes a leuco dye in the presence of peroxidase to produce a dye having a maximum absorption at 670 nm. The density of the dye, which is proportional to the concentration of cholesterol in the sample, is then measured by reflectance spectrophotometry.

b. Specimen Collection and Preparation: Analysis can be performed on serum or disodium EDTA-treated plasma. Although fasting is not a requirement, it has been suggested that a subject should fast for 12-14 hours before the sample is drawn.

Specimen Treatment: HDL separations should be made as soon as possible after specimens have been collected, preferably the same day. If separations must be delayed beyond the day of collection, samples can be stored at -15 °C for several weeks or at -60 °C for several months. In no case should samples be subjected to more than a single freeze-thaw cycle before being analyzed.

c. Test Reagents:

(1) Ingredients: Reactive ingredients are cholesterol oxidase, cholesterol ester hydrolase, peroxidase, and 2-(3,5-dimethoxy-4-hydroxyphenyl)-4,5-bis(4-dimethylaminophenyl) imidazole. Other ingredients are pigment, binders, surfactants, buffer, preservatives, and polymer cross-linking agent.

(2) Dextran sulfate stock solution: (20 g/L) (Dextralip 50, Mr 50,000±5,000 from Sochibo, SA, Boulogne, France 92100) Add 1.0 g of dextran sulfate to 40 ml of deionized water in a beaker and, if necessary, adjust the pH to 7.0 with dilute HCL. QS to 50 ml. A preservative solution containing per liter, 50 g of sodium azide (N_aN_3) (Catalog no. 52002) , 1.0 g of chloramphenicol) (Catalog no. C0378), and 0.5 g gentamicin sulfate (Catalog no. C3632) (all from Sigma Chemical Co., St. Louis, MO 63178) can be added at the rate of 10 ml/L before final volume adjustment of the stock solution. Store at 4 °C. Solution is stable for 4 months.

(3) Stock Mg2: Solution (1.0 mol/L) (Catalog no. M8266, Sigma Chemical Co., St. Louis. MO 63178). Add 10.15 g of $MgCl_2.6H_2O$ to 40 ml of deionized water in a beaker; adjust the pH to 7.0 with dilute NaOH. QS to 50 ml. Store at 4 °C. Solution is stable for 4 months.

(4) Combined Working Reagent: Mix equal volumes of the dextran sulfate and $MgCl_2$ stock solutions to produce a solution with a dextran sulfate concentration of 10 g/L and a $MgCl_2$ concentration of 500 mmol/L. Combined solutions are stable at 4 °C for 4 months.

Note: When making up new dextran sulfate stock and $MgCl_2$ stock, run four parallel runs before using.

(5) NaCl (0.15 mol/L): (Catalog no. S9625, Sigma Chemical Co., St. Louis, MO 63178). Add 0.42 g NaCl to 100 ml H_2O.

d. Test Procedure

Precipitation Step:

(1) Transfer 0.5 ml of participant and control samples to 10- X 75-mm tubes. (Specimen volumes can be adjusted as long as a proportionate volume of precipitant reagent is added.)

(2) Add 50 ul of the combined working reagent and vortex for 3 seconds. Let the tubes stand at room temperature for 10 minutes. Centrifuge for 15 minutes with a refrigerated centrifuge (4 °C) at 3,000 X g.

(3) Obtain an aliquot of the clear supernates and run the HDLC test on the Ektachem.

(4) To clear turbid supernates the following: Without separating the turbid supernate from the precipitate, add 0.5 ml of 0.15 mol/L NaCl solution and another 50 ul of combined working reagent. Mix, let stand 10 minutes. Centrifuge as above. Supernatant cholesterol must be multiplied by 2.

e. Results: Results should be multiplied by 1.1 to correct for the dilution factor 1.0 ml:1.1 ml.

f. References:

(1) Eastman Kodak. HDL cholesterol, Ektachem test methodology. Rochester, New York: Eastman Kodak, 1984.

(2) Warnick GR, Benderson J, Albers JJ. Dextran sulfate - Mg2+ precipitation procedure for quantitation of high density lipoprotein cholesterol. In: Cooper GR, ed. Selected methods of clinical chemistry. Washington DC: American Association for Clinical Chemistry, 1983;10:91-9.

16. CREATINE PHOSPHOKINASE (CK)

a. Test Principle: In the assay for creatine phosphokinase (CK), creatine phosphate and adenosine diphosphate (ADP) are used as substrates to generate creatine and adenosine triphosphate (ATP). In a coupled reaction sequence, a dye precursor is oxidized by hydrogen peroxide that is produced in stoichiometric equivalents to ATP formed in the initial reaction. The rate of chromophore production is monitored by reflectance spectrophotometry at 670 nm and used as a measure of creatine kinase activity.

b. Specimen Collection and Preparation: Analysis should be performed on a serum or plasma specimen that has been collected by standard venipuncture technique. For plasma specimens, heparin may be used as an anticoagulant.

Special Precautions: CK is unstable in serum. Serum should be promptly removed from the clots, and the specimen promptly analyzed.

Incompletely clotted samples containing fibrin materials can cause problems in the analyzer's metering.

c. Test Reagents:

Ingredients: Reactive ingredients are L-a-glycero-phosphate oxidase; peroxidase; ascorbate oxidase; glycerol kinase; creatine phosphate; N-acetylcysteine; magnesium acetate; glycerol; 2-(3,5-dimethoxy-4 hydroxphenyl)-4,5-bis (4-dimethylaminophenyl) imidazole; and adenosine diphosphate. Other ingredients are pigment, binder, buffers, surfactants, solvent, inhibitors, polymer cross-linking agent, and antioxidant.

d. Reference: Eastman Kodak. Creatine phosphokinase, Ektachem test methodology. Rochester, New York: Eastman Kodak, 1984.

17. SERUM CREATININE (SCREA)

a. Test Principle: The analysis is based on an enzymatic hydrolysis of creatinine to produce ammonia, and the selective migration of ammonia through a semipermeable membrane to a layer containing an indicator dye. The color in the indicator layer changes in proportion to the ammonia concentration and the change can be measured spectrophotometrically at 600 nm.

The presence of creatinine iminohydrolase results in the Ektachem slide (CREA) measuring both the ammonia generated from creatinine and the endogenous ammonia present in the specimen. The Ektachem slide (NH_3) measures only the endogenous ammonia. The dye density measurements from the two slides make

it possible to obtain a corrected measurement related only to ammonia generated from creatinine -- hence, a determination of the creatinine concentration.

b. Specimen Collection and Preparation: Analysis should be performed on a serum or heparinized plasma specimen (excluding ammonium heparin) that has been collected by standard venipuncture technique. Participants should be in a fasting state; the tourniquet should be applied so that the pressure it provides is minimal. Ammonium heparin and fluoride/oxalate must be avoided as anticoagulants.

Special Precautions: Since the Ektachem slides for creatinine ultimately quantitate ammonia, it is advantageous to minimize specimen ammonia as much as possible. For this reason, serum should be separated from contact with cells as soon as possible after clotting, either by thixotropic gels or by pouring off the serum into a clean tube. If analysis is delayed, separated serum should be refrigerated or frozen. The sample size is 10 ul.

c. Test Reagents:

(1) Ingredients: Creatinine, imohydrolase, bromphenol blue. Other ingredients: pigment, binders, surfactants, buffers, and humectant.

(2) Storage and Preparation: Cartridges of Ektachem clinical chemistry slides for creatinine and ammonia must be stored under conditions that will protect the slides from corrosive elements, high temperatures, and high humidity. Slide cartridges should be stored at 2-8 °C.

(3) Ektachem slides for creatinine and ammonia are affected by temperature and humidity over time. The following protocol has been derived to minimize these effects. Note: Precision and accuracy for creatinine as stated under Performance Characteristics are based on the use of this protocol.

(4) For creatinine calibration and analysis, Ektachem slides for creatinine and Ektachem slices for ammonia should be handled as a pair, i.e., fresh cartridges should be loaded together, removed from the analyzer together for desiccated refrigerated storage, reloaded together, etc.

(5) Cartridges containing slides for creatinine and ammonia can be removed from refrigeration and allowed to reach room temperature before being loading. To avoid condensation on the slide, remove the cartridge from its wrapper or other airtight container immediately before loading it into the analyzer.

(6) Unlike most Ektachem clinical chemistry slides, cartridges of creatinine and ammonia slides may not be stored on the analyzer for 1 week. Creatinine and ammonia slides are stable on the analyzer for a maximum of 48 hours. If, however, laboratory temperature and humidity exceed 24 °C (75 °F)/55% relative humidity, normal quality controls should be run (in duplicate) every 8 hours to verify the continued validity of results. Examine for upward drift (exceeding 0.1 mg/dl) by averaging and comparing with the results from fresh slides. If creatinine and ammonia slides have not been exhausted within 48 hours, they should be replaced with fresh slides.

(7) If creatinine assays are not being run frequently, or if the analyzer will be shut down, creatinine and ammonia slides should be removed from the analyzer's slide supplies. Partially used cartridges should be stored desiccated and refrigerated and returned to the analyzer during testing periods. Place partially used cartridges in a dry, airtight container and return them to refrigerated storage. A round, widemouth, glass bottle with a tight-sealing screwcap is a suitable container for at least 2 weeks of refrigerated storage. The bottle should be of minimum size to provide the least air space. Such bottles are available from most laboratory supply dealers.

(8) A small amount of desiccant (granular silica gel) should be placed in the container to protect the slides from moisture. Provide tissue or some other suitable barrier to prevent desiccant granules from contaminating the slides.

(9) When reloading partially used cartridges on the analyzer, observe the total, on-analyzer limits of 48 hours.

(10) Slides should be used before the date on the cartridge box.

(11) A fresh cartridge of creatinine and ammonia slides should always be used for calibration.

(12) Cartridges may be loaded directly from refrigerated storage; however, the first slide in each cartridge must be discarded after loading to avoid condensation on the slide. To discard the first slide, run Option 7 or, if no other cartridges of ammonia or creatinine slides are on the analyzer, a water or waste cup may be run.

d. Reference: Eastman Kodak. Creatinine test, Ektachem test methodology. Rochester, New York: Eastman Kodak, 1985.

18. URINE CREATININE (UCREA)

a. Test Principle: Creatinine determinations are made by depositing a drop (10 ul) of the specimen on the Ektachem creatinine slide and a second drop on the Ektachem NH_3 slide. The slides differ in that the ammonia slide contains no creatinine iminohydrolase.

Each slide has a spreading layer to meter and spread the sample uniformly and to allow water and nonpoteinaceous components to pass to the underlying reagent layer. The reagent layers of the two slides differ. Creatinine iminohydrolase in the creatinine slide catalyzes the hydrolysis of creatinine to produce N-methylhydantoin and ammonia. No ammonia is generated from the creatinine in the NH_3 slide; only the free ammonia from the specimen is measured. The ammonia in each slide then diffuses through the semipermeable membrane to react with an ammonia indicator.

After a fixed incubation time, the reflection density of the dye in each slide is measured spectrophotometrically at 600 nm through the transparent support, with the white background of the spreading layer used as a diffuse reflector. Both creatinine and ammonia values are reported out.

Because of the typically high concentration of ammonia found in urine specimens, the concentration of ammonia in the specimen must be reduced by treating the specimen with an ion exchange resin. The resin removes ammonia as the ammonium ion.

b. Specimen Collection and Preparation: Analysis is performed on timed or random urine specimens collected by standard laboratory procedures.

(1) Properly treat the urine specimens with resin or store them refrigerated or frozen to minimize ammonia accumulation.

(2) Resin-treated urine removed from the resin beads is stable several days at refrigerated temperatures.

c. Test Reagents:

(1) Urine Creatinine Buffer: Tetraborate buffer solution used for diluting the urine and for providing the optiman pH for removal of ammonia as the ammonium ion. Unopened bottles are stored at 2-8 °C. Opened bottles are stored at room temperature and should be discarded after 3 months.

(2) Resin Tubes: Tubes containing ion-exchange resin to reduce NH_3 concentration, manufactured by Kodak, stored at room temperature.

(3) Cartridges of Ektachem clinical chemistry slides for creatinine and ammonia must be stored under conditions that will protect the slides from corrosive elements, high temperature, and high humidity. Slide cartridges should be stored in a refigerator at 2-8 °C.

(4) Ektachem slides for creatinine and ammonia are affected by temperature and humidity over time. The following protocol has been established to minimize these effects. Note: Precision and accuracy for creatinine as stated under Performance Characteristics are based on this protocol.

(5) For creatinine calibration and analysis, Ektachem slides for creatinine and Ektachem slides for ammonia should be handled as a pair, i.e., fresh cartridges should be loaded together, removed from the analyzer together for desiccated refrigerated storage, reloaded together, etc.

(6) Cartridges containing slides for creatinine and ammonia can be removed from refrigeration and allowed to reach room temperature before being loaded into the analyzer. To avoid condensation on the slide, remove the cartridge from its wrapper or other airtight container immediately before loading it.

(7) Unlike most Ektachem clinical chemistry slides, cartridges of creatinine and ammonia slides may not be stored on the analyzer for 1 week. Creatinine and ammonia slides are stable on the analyzer for a maximum of 48 hours. If, however, laboratory temperature and humidity exceed 24 °C (75 °F)/55% relative humidity, normal quality controls should be run (in duplicate) every 8 hours to verify continued validity of results. Examine results for upward drift (exceeding 0.1 mg/dl) by averaging and comparing with the results from fresh slides. If creatinine and ammonia slides have not been used within 48 hours, they should be replaced with fresh slides.

(8) If creatinine assays are not being run frequently, or if the analyzer will be shut down, creatinine and ammonia slides should be removed from the analyzer's slide supplies. Partially used cartridges should be stored desiccated and refrigerated and returned to the analyzer during the testing period. Place partially used cartridges in a dry, airtight container and return them to refrigerated storage. A round, widemouth glass bottle, with a tight-sealing screwcap, is a suitable container for at least 2 weeks of refrigerated storage. The bottle should be of minimum size to provide the least air space. Such bottles are available from most laboratory supply dealers.

(9) A small amount of desiccant (granular silica gel) should be placed in the container to protect the slides from moisture. Provide tissue or another suitable barrier to prevent desiccant granules from contaminating the slides.

(10) When reloading partially used cartridges on the analyzer, observe the total, on-analyzer limits of 48 hours.

(11) Slides should be used before the date on the cartridge box.

(12) A fresh cartridge of creatinine and ammonia slides should always be used for calibration.

d. Test Procedure: Gently tap each tube to dislodge any resin beads that may have adhered to the top of the tube.

(1) Add 1.0 ml of urine creatinine buffer to the resin tube and swirl gently. Use Accuprep to dispense 1 ml. Use a 50-ul MLA pipet to dispense patient samples.

(2) Add 50 ul of urine to the tube containing the resin and buffer. CAUTION: DO NOT ADD URINE TO TUBES BEFORE ADDING BUFFER or the creatinine will adsorb onto the resin beads. Recap with the original cap.

(3) Put the tube on a rocker for 10 minutes to mix the contents.

(4) Remove tube from rocker and wait for the beads to settle.

(5) Immediately pipet the diluted specimen off of the resin beads. Be careful not to aspirate any resin beads. CAUTION: SPECIMEN MUST BE REMOVED FROM THE RESIN WITHIN 10 MINUTES AFTER THE BEADS SETTLE.

(6) Run specimen on the Ektachem 700, remembering to enter a dilution factor of 21. The instrument will automatically calculate the dilution.

e. Reference: Eastman Kodak. Creatinine, Ektachem test methodology. Rochester, New York: Eastman Kodak, 1985.

19. GAMMA-GLUTAMYLTRANSFERASE (GGT)

a. Test Principle: In the assay for gamma-glutamyltransferase (GGT), the enzyme catalyzes the transfer of the gamma-glutamyl moiety from the donor substrate, a gamma-glutamyl peptide, to an acceptor substrate, an amino acid, or another peptide. The gamma-glutamyl group is transferred from gamma-glutamyl-p-nitroanilide to an amino acid acceptor, glycylglycine. The rate of release of p-nitroaniline is used as a measure of GGT activity.

b. Specimen Collection and Preparation: Analysis should be performed on a serum or plasma specimen that has been collected by standard venipuncture technique. Heparin and EDTA may be used as anticoagulants for plasma specimens.

Special Precautions: Serum should be removed promptly from the clot. Hemolyzed specimens should not be used. Incompletely clotted samples containing fibrin materials can cause problems in the analyzer's metering. If analysis cannot be performed immediately, the specimen should be refrigerated or frozen. Specimens should be frozen for long-term storage or shipment. The sample size is 11 ul.

c. Test Reagents:

Ingredients: Reactive ingredients are glycylglycine and L-gamma-glutamyl-p-nitroanilide. Other ingredients are polymers, binders, and surfactant.

d. Reference: Eastman Kodak. Gamma-glutamyltransferase, Ektachem test methodology. Rochester, New York: Eastman Kodak, 1984.

20. FASTING GLUCOSE (GLU)

a. Test Principle: The method of analysis is an adaptation of the glucose oxidase-peroxidase-chromogen coupled system for glucose determination in biologic fluids. Hydrogen peroxide is produced in the oxidation of glucose catalyzed by glucose oxidase. The peroxide acts as oxidant in a peroxidase-catalyzed oxidative-coupling reaction to form a dye.

b. Specimen Collection and Preparation: Analysis should be performed on a serum or plasma specimen collected by standard venipuncture technique. No special patient preparation is necessary for routine glucose measurements.

Special Precautions: Serum must be removed promptly from the clot keep the cells from metabolizing glucose (about 7% per hour at room temperature). Hemolyzed specimens must not be used. The sample size is 10 ul.

If the analysis cannot be performed immediately, properly collected specimens may be refrigerated at 4 ^{o}C for up to 3 days. Specimens should be frozen for long-term storage or shipment.

c. Test Ingredients: Ingredients are glucose oxidase (source: Aspergillus niger); peroxidase (source: horseradish); 1,7-dihydroxynapthalene and 4-aminoantipyrine hydrochloride. Other ingredients are pigment, binders, buffers, surfactants, antioxidants, and a polymer cross-linking agent.

d. Reference: Eastman Kodak. Glucose analysis, Ektachem test methodology. Rochester, New York: Eastman Kodak Company, 1985.

21. LACTATE DEHYDROGENASE (LDH)

a. Test Principle: The assay for lactate dehydrogenase (LDH) uses pyruvate and NADH as substrate to generate lactate and NAD+. The oxidation of NADH, which is monitored by reflectance spectrometry at 340 nm, is used as a measure of lactate dehydrogenase.

b. Specimen Collection and Preparation: Analysis should be performed on a serum or plasma specimen that has been collected by standard venipuncture technique. Heparin may be used as an anticoagulant for plasma specimens.

Note: Heparin plasma analyzed for LDH on the Ektachem 700 analyzer has been shown to produce results similar to those for serum. Some other methods, however, have been reported to show substantial differences between results for heparin plasma and serum specimens. This problem is reportedly due to contamination by platelets if the plasma is separated by low speed centrifugation. Therefore, results for heparin plasma specimens obtained with such methods and with the Ektachem analyzer may not agree.

Special Precaution: Serum should be removed promptly from the clot. Hemolyzed specimens should not be used since LDH from red cells will contaminate the specimen.

Since LDH activity in serum specimens is relatively unstable, serum specimens should be analyzed promptly. DO NOT FREEZE. The sample size is 11 ul.

c. Test Reagents

Reactive ingredients are nicotinamide adenine dinucleotide, reduced; and sodium pyruvate. Other ingredients are polymer beads, binders, buffer, surfactants, polymer cross-linking agent, and preservative.

Note: The dynamic range of the LDH assay is directly proportional to the amount of NADH available in the slide. Since NADH is sensitive to temperature, humidity, and light, any excessive or prolonged environmental exposure could result in an elevated sample (yet still within the dynamic range) being flagged by the analyzer as requiring dilution. Therefore, slide storage and handling conditions must be adhered to.

d. Reference: Eastman Kodak. Lactate dehydrogenase, Ektachem test methodology. Rochester, New York: Eastman Kodak, 1985.

22. TOTAL PROTEIN (TP)

a. Test Principle: The method of analysis is based on the well-known biuret reaction, characterized by the generation of a violet-colored complex when protein is treated with cupric ion (Cu^{2+}) in an alkaline medium. The density of the resulting complex is related to the concentration of total protein in the sample and can be measured spectrophotometrically.

b. Specimen Collection and Preparation: Analysis should be performed on a serum specimen that has been collected by standard venipuncture technique. Participants should be in a fasting state; the tourniquet should be applied so that the pressure it provides is minimal. Lithium heparin may be used as an anticoagulant for plasma specimens.

c. Test Reagents:

Ingredients: Copper sulfate, tartaric acid, acid lithium hydroxide. Other ingredients: binders, surfactants, and polymer cross-linking agent.

d. Reference: Eastman Kodak. Total protein, Ektachem test methodology. Rochester, New York: Eastman Kodak, 1985.

23. TRIGLYCERIDE (TRIG)

a. Test Principle: The analysis is based on a totally enzymatic method. In the method, an enzyme, L-2-glycerophosphate oxidase, is used to catalyze the oxidation of L-2-glycerophosphate to generate hydrogen peroxide. The peroxide oxidizes a leuco dye in a reaction catalyzed by peroxidase to produce a dye having a maximum absorption at about 660 nm. The density of the dye is related to the concentration of triglycerides in the sample and can be measured spectrophotometrically.

b. Specimen Collection and Preparation: Analysis should be performed on a serum specimen that has been collected by standard venipuncture technique. Participants should be in a fasting state for at least 12, preferably 16, hours before the sample is drawn. Lithium heparin and EDTA may be used as anticoagulants for plasma specimens.

Special Precautions: The stability of triglycerides at room temperature is reported to be 1 to 6 days, with a 0% to 10% decrease. Refrigerated or frozen specimens would be expected to have longer stability. Repeated freeze/thaw cycles may result in decreased triglyceride solubility. The sample size is 10 ul.

c. Test Reagents:

Ingredients: Lipase M, peroxidase, ascorbate oxidase, glycerol kinase, L-2-glycerophosphate oxidase, 2-(3,5-dimethoxy-4-hydroxphenyl) 4,5-bis(4-dimethylaminophenyl) imidazole, adenosine triphosphate, magnesium chloride. Other ingredients are pigment, binder, buffer, surfactants, solvent, antioxidants, inhibitors, and polymer cross-linking agent.

d. Reference: Eastman Kodak. Triglyceride test, Ektachem test methodology. Rochester, New York: Eastman Kodak, 1985.

V. ANALYTICAL METHODS

K. Delta-Aminolevulinic Acid (D-ALA) in Serum
Manual Colorimetric Method

1. Principle

D-ALA is an intermediate in the series of reactions that lead to porphobilinogen, porphyrins, and heme. Alterations in the heme biosynthetic pathway as a result of lead poisoning, liver disease, or other conditions may lead to a decrease in ALA dehydratase in erythrocytes and other cells and a subsequent increase in D-ALA levels in serum.

In this procedure, interfering thiol compounds present in serum are eliminated by adding iodoacetamide. Trichloroacetic acid is used to obtain protein-free serum. Subsequent heating in the presence of acetylacetone results in the formation of a pyrrole derivative of D-ALA. This compound is reacted with Ehrlich's reagent and measured spectrophotmetrically.

2. Specimen

a. No preparation of the participant is needed.

b. Only serum is suitable for this test. Blood is collected in a 15-ml Vacutainer, allowed to clot, centrifuged and separated from the cells within 1 hour of its being collected.

c. D-ALA is stable at 2-8 °C for 24 hours. For longer storage, freeze at -20 °C.

d. A minimum of 3 ml of serum is required for this assay. Serum should be free of hemolysis, lipemia, or icterus.

3. Reagents, Supplies, and Equipment

a. Glass test tubes, disposable, 16 X 100 mm

b. Pipets: MLA with disposable tips, 0.5 ml, 1 ml
Beckman Accuprep (Refer to operations manual before using.)
Eppendorf repeat pipet with disposable Combitips, 12.5 ml, 5 ml, 2.5 ml

c. Volumetric flasks (Class A), 1 L, 100 ml, 50 ml, 10 ml

d. Analytical balance, Mettler AE 166

e. Timer

f. Centrifuge, Isodata 3E-1

g. Serum separators to fit 16- X 100-mm tubes

h. Vortex mixer

i. Boiling water bath

j. Spectrophotometer with 1-2 mm band width (Beckman DU-7) (Refer to operation manual before using.)

k. Test-tube racks

l. Magnetic stirrer

m. Repipets: 2.5 ml, 5 ml, 1 ml

n. Plastic caps, 16 mm

o. Foil

p. Iodoacetamide, 0.06M

(1) Iodoacetamide, 120 mg

Reagent grade, Fisher. Store at 2-8 °C. Reagent water, type I; QS to 10 ml

(2) After dilution, label as "Iodoacetamide, 0.06M" with the date, technician's initials, and expiration time of 8 hours. Store at room temperature.

q. Trichloroacetic Acid, 200 g/L

(1) Dissolve:
(a) Trichloroacetic acid, 200 g
Certified ACS, Fisher.
(b) Reagent water, type I; QS to 1 L

(2) Transfer to brown bottle equipped with a 1-ml repipet, label as "Trichloroacetic Acid; 200 g/L" with preparation date, technician's initials, and an expiration date of 1 year. Store at room temperature.

r. Acetate Buffer, pH 4.6

(1) Dissolve in a 1-L volumetric flask:
(a) Acetic acid, glacial; 57 ml
Analytical grade, Mallinckrodt.
(b) Sodium acetate trihydrate; 136 g
Laboratory grade, Fisher
(c) Reagent water, type I; QS to 1 L

(2) Verify pH at 4.6, adjust if necessary (using HCl and NaOH as needed).

(3) Bottle, label as "Acetate Buffer, pH 4.6" with preparation date, technician's initials, and an expiration date of 1 year. Store at room temperature.

s. Acetylacetone, reagent grade, Fisher. Store in nonflammable cabinet.

t. Ehrlich's Reagent:

(1) Combine in a 250-ml brown glass bottle.
(a) P-dimethylaminobenzaldehyde; 2.0 g
Sigma, PROTECT FROM LIGHT
(b) HCl, concentration 25 ml
reagent grade ACS; MCB reagents.
(c) Glacial acetic acid, 75 ml,
analytical grade, Mallinckrodt

(2) Label as "Ehrlich's Reagent" with the date and time of preparation, technician's initials, and an expiration time of 6 hours. Store at room temperature.

u. Sodium Hydroxide, 2.5M

(1) Dissolve in a 1-L volumetric flask:
(a) Sodium hydroide, 100 g
reagent ACS, MCB reagents.
(b) Reagent water, type I QS to 1 L

(2) Label as "Sodium Hydroxide, 2.5M" with the preparation date, technician's initials, and an expiration date of 1 year. Store at room temperature.

v. Delta aminolevulinic acid HCl, Porphyrin Products, Logan, Utah. Store in refrigerator.

4. Calibration

a. D-ALA 1,000 ug/ml stock standard:

(1) Combine in a 100-ml volumetric flask
(a) D-ALA, 100 mg
(b) Reagent water, type I; QS to 100 ml

(2) When dissolved, freeze at -20 ^{o}C in 1-ml aliquots. Label with the preparation date, technician's initials, and an expiration date of 3 months.

b. D-ALA 10 ug/ml working standard:

Using a 500-ul volumetric pipet, deliver 0.5 ml of the 1,000 ug/ml stock standard to a 50-ml volumetric flask, and QS with distilled water. Prepare fresh daily.

c. Working standards at levels of 0.2, 0.3, 0.4, 0.5, 1.0, and 2.0 ug/ml D-ALA are prepared fresh daily and are included in each run. In preparing the standards, the following table may be used:

D-ALA Stds. ug/ml	Dilution	Mls of 10 ug/ml D-ALA	Type I Water	Total Volume (Ml)
0.2	1:50	0.2	9.8	10.0
0.3	1:33.3	0.3	9.7	10.0
0.4	1:25	0.4	9.6	10.0
0.5	1:20	0.5	9.5	10.0
1.0	1:10	1.0	9.0	10.0
2.0	1:5	2.0	8.0	10.0

Standards are run in duplicate on each analytical run. Use the Accuprep to dispense the indicated amounts of the 10 ug/ml standard into 10-ml volumetric flasks and QS with type I water.

5. Quality Control Material

The control material consists of a serum (bovine or human) spiked with D-ALA. The control is run in duplicate at the beginning and end of each analytical run. The control is prepared by adding 180 mg D-ALA stock standard (1,000 ug/ml) to 1 L of bovine serum. The contol material is frozen in 3-ml aliquots and stored at -20 °C. When stored at this temperature, it is stable for 6 months.

To prepare the D-ALA control, add 180 ul of D-ALA stock standard to 1 L of bovine serum (Colorado Senum Co.). (Use the Accuprep for the additional stock standard.)

6. Procedure

a. Allow all reagents, standards, and participant specimens to come to room temperature before testing.

b. Label one 16- X 100-mm test tube for each participant and control sample. Place 3.0 ml of sample (use a 1- to 5-ml adjustable MLA pipet) in the labeled tube. Using an Eppendorf pipet, add 0.2 ml of 0.06M iodoacetamide. Vortex thoroughly and let set 2 minutes at room temperature.

c. Using the repipet, add 1.0 ml of trichloroacetic acid to each tube. Cap each tube and shake vigorously. Centrifuge for 15 minutes at 3500 rpm. Use serum separator to avoid pipetting any precipitate during testing. Insert separator into top of precipitate.

d. Label three 16- X 100-mm tubes for each participant and control sample (two tubes for test duplicates, and one for a serum blank.) Label two tubes for each standard.

e. Make standards, following directions in calibration section.

f. Using a 0.5-ml MLA, pipet 0.5 ml of the supernatant obtained from the TCA addition and centrifugation to each tube. Add 0.5 ml of standard to each standard tube.

g. Prepare six reagent blanks by adding 0.5 ml type I water to 6 labeled tubes.

h. Use the Eppendorf pipet set to 0.5 ml, to dispense 0.5 ml of acetate buffer. Vortex. Add 0.05 ml of 2.5M NaOH to each tube. Vortex. Add 0.025 ml of acetylacetone with the Accuprep to each tube, EXCEPT those used for the serum blanks.

i. Vortex. Cover the tubes tightly with foil, making an indentation in the top of the foil to aid condensation. Place the tubes in a boiling water bath for 10 minutes. Cool to room temperature. Vortex.

j. Using a 500-ul MLA pipet, add 0.5 ml of this solution to labeled 16- X 100-mm test tubes. Using the Eppendorf pipet set to 0.5 ml, add 0.5 ml of Ehrlich's reagent to each tube. Vortex.

j. After exactly 15 minutes, read the absorbance at 553 nm against the reagent blank. The serum blank absorbances should be recorded and subtracted from the test absorbances for each participant and control sample.

7. Calculations

a. Subtract participant blank absorbance from the test absorbance.

b. Use the linear regression program in the CHC or the IBM PC/XT computer to evaluate the standard curve and obtain patient results.

c. Participant and control results are obtained from the standard curve. After of this result has been verified, use the force calculations mode to perform the following calculation:

$$\underset{(ug/dl)}{\text{D-ALA}} = \underset{(ug/ml)}{\text{Uncorrected D-ALA}} \times 1.4 \times 100$$

8. Reference

Labbe, RF. Porphyrins and related compounds. In: Tietz NW, ed. Fundamentals of clinical chemistry. Philadelphia: WB Saunders, 1976:469-71.

V. ANALYTICAL METHODS

L. Glycerol in Serum
(Abbott - VP)

1. Principle

Usually, glycerol concentrations in serum or plasma are low, but if the specimens are improperly stored or are contaminated with glycerol as they are being handled, the concentrations may be higher. In these circumstances, the corrected triglyceride concentration should be measured.

In this assay, endogenous glycerol in the participant sample initiates a series of enzymatic reactions that lead to the oxidation of NADH, which is measured photometrically. Subtracting the measured free glycerol from the Ektachem total triglyceride result gives an absolute measurement of the triglyceride present.

2. Specimen

Serum only. Separate as soon as possible after collection. Specimen should be free of hemolysis. Minimum test volume is 100 ul.

3. Reagents, Supplies, and Storage

a. Abbott, A-Gent Triglyceride Blank Reagent, (10 X 21 ml) FOR RESEARCH USE ONLY.

(1) Lyophilized form stored at - 20 °C is stable for up to 2 years.

(2) Reconstitute with 21 ml of deionized H_2O, using a repipet or the Beckman Accuprep. The reconstituted solution is stable for 24 hours at 2-8 °C.

b. Equipment:

(1) Abbott VP Biochromatic Analyzer
(2) Multicuvettes
(3) Microcups
(4) 100-ul MLA pipet
(5) Accuprep with 10-ml syringe
(6) 1.0-ml class A volumetric pipets

4. Calibration

Two working standards are used in the glycerol assay with concentrations of 5 and 25 mg/dl. Working standards are prepared from a previously prepared 1,000 mg/dl stock standard of glycerol and a 100 mg/dl working standard.

a. Preparation of stock glycerin - 1,000 mg/dl
(1) Combine in a 50-ml class A volumetric flask:

(a) Glycerin ($C_3H_8O_3$) 99.5%
Fisher Scientific catalog no. 6-33, 0.5025 g
(b) Reagent water type I, QS to 50 ml

(2) Label as "Stock Glycerin 1,000 mg/dl" with the date of preparation, technician's ID, and an expiration date of 6 months. Stopper tightly and seal with parafilm.

(3) Label should include the name of the standard, preparation date, expiration date, and the technologist's initials.

(4) Store at 2-8 °C. Solution is stable for six months.

b. Preparation of working standards:

(1) 100 mg/dl standard:
Combine in a test tube:

(a) 1,000 mg/dl stock standard
9.0 ml (Accuprep 10-ml syringe)
(b) Reagent water type I
1.0 ml (Class A volumetric pipet)

(i) Mix well. Label as "100 mg/dl Glycerol Standard" with technician's initials and date of preparation.
(ii) Stable 8 hours at 2-8 °C. Prepare fresh daily.

(2) 25 mg/dl standard:
Combine in a test tube:

(a) 100 mg/dl working standard
1.0 ml (class A volumetric pipet)

(b) Reagent water type I
3.0 ml (Accuprep 10-ml syringe)

(i) Mix well. Label as "25 mg/dl Glycerol Standard" with technician's initials and date of preparation.
(ii) Stable 8 hours at 2-8 °C. Prepare fresh daily.

(3) 5 mg/dl standard:
Combine in test tube:
(a) 25 mg/dl working standard
1.0 ml (class A volumetric pipet)

(b) Reagent water type I
4.0 ml (Accuprep 10-ml syringe)

(i) Mix well. Label 5 mg/dl glycerol standard with technician's initials and date of preparation.
(ii) Stable 8 hours at 2-8 °C. Prepare fresh daily.

5. Quality Control Material

   a. Chemtrak controls, levels I and III. Manufactured by Medical Analysis Systems, Inc. Unopened vials of ChemTrak are stable until the expiration date on the label when stored frozen at -10 to -20 °C. Bacterial contamination produces an increase in turbidity, a characteristic odor, and/or a decrease in the glucose level. Discard vial if any of these phenomena are observed.

   b. To prepare control, thaw unopened vials by rotating approximately 1 hour at room temperature. Shake vigorously before each use. Aliquot 1 ml into plastic vials. Store at -10 to -20 °C. Thaw vials just before use and rock for 15 minutes.

   c. If tolerance limits are exceeded, take corrective action before allowing the analysis is allowed to continue.

6. Procedure

   a. Press power to turn instrument on. The instrument must warm up 15-20 minutes before the temperature check. After about 10 seconds, press the C key.

   b. Perform daily maintenance and temperature check (Section 9.9 in Abbott VP Manual).

   c. Fill incubator with 28 ml of degassed deionized water. Do not overfill but be certain that the water is at least 1/16 inch above the lens.

   d. Reconstitute the reagent for triglyceride blank reagent; add 21 ml of deionized water.

   e. Install (340/380) filter (if it is not in place).

   f. Insert a clean multicuvette and the number of sample cups needed for a run.

   g. Prime instrument with reagent by pressing 002 E.

   h. Load tray in the following order:

      00 - No cup
      B1 - Deionized water
       2 - 5 mg/dl standard
       3 - 25 mg/dl standard
       4 - Control
       5 - Participant sample
       6 - Etc.

      Note: Pipette aqueous samples (standards) last to minimize evaporation effects. Minimum sample size is 100 ul.

i. After all samples have been poured, press test number 97, E.

j. After VP has pipetted cup 00, watch digital display for optical density (OD) reading of cup 01. Record values on reagent blank log. Value must be greater than 1.5.

k. After the last run of the day has been completed, press 00 1 E, 3E, to wash the lines with water. Discard water incubator and dry with a Kimwipe. Turn the power off.

7. Calculations

a. The glycerol blank value obtained from the VP analyzer must be corrected by multiplying the blank value by 9.6. This factor is derived from the ratio of the molecular weights of glycerol and triolein.

Glycerol blank mg/dl X 9.6 factor = corrected glycerol (in mg/dl).

b. Once the corrected glycerol value has been calculated, it is subtracted from the total triglyceride value obtained from the Ektachem to achieve an absolute triglyceride value. The absolute triglyceride is obtained by computer, using the formula:

Total Triglyceride - Corrected Glycerol = Absolute Triglyceride

8. References

a. Abbott VP BiChromatic analyzer operation manual. North Chicago, Illinois: Abbott Laboratories, 1980.

b. Calbiochem-Behring. Enzymatic triglycerides-glycerol (3 vial) stat-pack [package insert]. LaJolla, California: Calbiochem-Behring, 1981; DOC L03092.

c. DuPont. ACA test methodologies, TGL, triglyceride. Wilmington, Delaware: E.I. duPont de Nemours & Co., 1982; PN 703772C.

V. ANALYTICAL METHODS

M. Cortisol in Serum
Aria HT

1. Principle

In the Aria HT cortisol system, diluted serum is incubated under conventional conditions, in the presence of radioactive (labeled) antigen and an antibody specific for cortisol to attain an equilibrium between nonbound antigen (labeled and unlabeled) and bound antigen (labeled and unlabeled). This incubation mixture then flows through a solid-phase elutable absorbent. The labeled and unlabeled cortisol not bound to the soluble antibody during incubation binds to the antibody chamber while the labeled and unlabeled cortisol bound to the soluble antibody during the incubation passes through the antibody chamber and into the bound flow cell where the labeled cortisol molecules are counted by a gamma detector. An eluting agent then passes through the antibody chamber to free the bound cortisol. The cortisol (labeled and unlabeled) freed from the antibody chamber by the eluting solution flows into the free flow cell where it is counted. Following this elution, the antibody chamber is rinsed and made ready for reuse in the next cycle.

Automation of this technique provides a high degree of uniformity of separation conditions.

Cortisol is a C-21 steroid whose secretion from the adrenal cortex, along with that of corticosterone and 11-deozycorticol, is regulated primarily by adrenocorticotrophic hormone (ACTH) acting via cyclic AMP.

Cortisol is carried in the circulation bound to a liver alpha-globulin called either transcortin (TC) or corticosteroid-binding globulin (CBG). It exerts widespread effects on the metabolism of proteins, on antibody synthesis in lymphoid tissue and on protein synthesis at the gene level. It is also involved in the metabolism of carbohydrates and lipids.

Cortisol is the most abundant corticosteroid circulating in human plasma and has been shown to serve as an indicator of adrenocortical function. Measurement of plasma cortisol levels is useful in the diagnosis and treatment of persons with Cushing's syndrome, Addison's disease, adrenal tumor, and other conditions related to the function of the adrenal cortex.

2. Specimen

a. Use only particulate-free serum. Plasma from blood anticoagulated in heparin is also acceptable.

b. A minimum volume of 50 ul is required for a single assay.

c. If specimens are not to be used immediately, cover with Parafilm and place in refrigerator. If necessary, the sample may be stored at 2-8 °C before analysis. If storage will exceed 24 hours, the sample should be frozen at -20 °C or lower. Cap the specimen containers when they are not in use.

d. Make sure that participant samples contain no exogenous radioactivity, since it may lead to erroneous results.

3. Reagents, Supplies and Storage

a. Cortisol Separation Chamber, catalog no. 622109, contains a solid-phase adsorbent. Store at room temperature in scintillation vial containing 10 ml of adsorption buffer 1 or 5.

Stability: A binding on the chamber test of greater than 88% with a 40% suppression in binding in the standard curve (e.g., a difference in binding of 40% between the percent binding of the lowest and highest concentrations) indicates a viable chamber. Replace when back pressure attributed to the chamber is greater than the level 1 alert (alert 48).

b. Cortisol Prefilter, catalog no. 600628, contains filtering material. Store at room temperature. Replace prefilter when back pressure exceeds the level 1 alert (alert 48).

c. 125-I-Cortisol Tracer, Cat.# 623890, contains 125-I-cortisol in phosphate buffer with 0.03% 8-anilino-1-naphthalene sulfonic acid (ANS). Each vial contains <10 microcuries (370 kBq) of 125-I-cortisol. Volume is 25 ml. Refrigerate at 2-8 °C. Refer to expiration date on vial.

d. Cortisol standards, catalog no. 623792, contains cortisol in stabilized human serum with 0.1% sodium azide and other preservatives.

Standard	Cortisol Amount	Volume	No. Vials
0	0 ug/dl	10 ml	2
A	2.0 ug/dl	5 ml	1
B	4.0 ug/dl	5 ml	1
C	8.0 ug/dl	5 ml	1
D	16.0 ug/dl	5 ml	1
E	32.0 ug/dl	5 ml	1
F	64.0 ug/dl	5 ml	1

Store at 2-8 °C. Refer to expiration date on vial. Do not use if turbid.

e. Sample Buffer 14, catalog no. 621803, aqueous solution containing cortisol antiserum (rabbit), TRIS, bovine serum albumin, ethanol (denatured), and 0.1% sodium azide as a preservative. Volume is 25 ml.

f. Adsorption Buffer, catalog no. 601128, aqueous solution containing phosphate, sodium chloride, and 0.02% sodium azide as a preservative. Volume is 2.0 L. Store at room temperature Refer to expiration date on bottle.

g. Elution Buffer 7, catalog no. 627518, aqueous acetonitrile solution containing glycinate. Volume is 2.0 L. Store at room temperature. Refer to expiration date on bottle.

h. Sample Buffer 1, catalog no. 604518, aqueous solution containing phosphate, citrate, and preservatives. Volume is 800 ml. Store at room temperature. Refer to expiration date on bottle. Do not use if turbid.

i. Rinse Solution 2, catalog no. 607614, stabilized distilled water with 0.02% sodium azide as a preservative. Volume is 2.0 L. Store at room temperature. Refer to expiration date on bottle.

j. Gamma cocktail, catalog no. 627119, contains alkyl imidazolinium dicarboxylate salt. Weight is 38 g. For use, mix the bottle well, add the concentrate to 3.8 L of distilled water and mix together well. Avoid contact with skin. Store at room temperature. The concentrate is stable until the expiration date stated on the vial. The diluted reagent is usable for 4 weeks after being formulated.

   Note: Gamma cocktail concentrate must be mixed before being diluted.

k. Sample Handler Rinse 2, catalog no. 645419, aqueous solution containing TRIS, bovine serum albumin, sodium chloride, surfactant, and preservatives. Volume is 2.0 L. Store at room temperature. Refer to expiration date on bottle.

l. LiquiCap, catalog no. 642312, contains polydimethylsiloxane. Volume is 35 ml. Store at room temperature. Refer to expiration date on bottle.

4. Calibration

a. Volume of standard dispensed depends on the number of samples to be assayed. The ARIA HT will restandardize as programmed by the operator. The chamber binding has been demonstrated to be stable, ±3%, up to 100 cycles. Each restandardization is performed from the same sample cups:

Volume to Dispense	No. of Aspirations
At least 50 ul	1-3
At least 65 ul	4
At least 75 ul	5

b. Add the required amount of standard to each of the following sample cups:

Standard	Cup
0.0 ug/dl	1
2.0 ug/dl	2
4.0 ug/dl	3
8.0 ug/dl	4
16.0 ug/dl	5
32.0 ug/dl	6
64.0 ug/dl	7

5. Quality Control Material

Two levels of Lyphochek controls, levels II and III, will be run in duplicate, one level each at the beginning and end of each analytical run.

To reconstitute the product, add 5.0 ml of type I water with a class A volumetric pipet to the desired vials. Replace the rubber stopper and allow the reconstituted material to stand at least 15 minutes. After that time, invert the bottle several times to ensure that the contents are thoroughly mixed. Inspect each vial carefully for undissolved material before initial use. Reconstituted controls should be stored at 2-8 °C and are stable for 7 days.

6. Procedure

a. All reagents and samples must be brought to room temperature before they are used. To keep them from deteriorating, do not leave them at room temperature longer than necessary.

b. The standard curve is run by using single standard level points. Participant samples should be run after establishing a standard curve.

c. Dispense the appropriate volume of standards in cups 1-7.

d. Dispense enough zero standard in the first cup before dispensing the control and participant samples to aspirate enough for the programmed warm-ups.

e. Add a minimum of 50 ul of the control or participant samples to the appropriate cups.

f. Overlay all standards, controls and participant samples with 50 ul of LiquiCap. Make sure that the LiquiCap is dispersed by tipping the tray.

g. For instrument setup instructions, the Aria HT Instruction Manual.

7. Calculation

By means of an iterative technique, the HT computer determines certain parameters used in the sample concentration determination. For the definite calculations, see the Aria HT Instruction Manual.

8. Reference

a. Becton Dickinson. Instruction manual for determination of cortisol using the Aria HT. Orangeburg, New York: Becton Dickinson, 1985; RMNo. 71411.

V. ANALYTICAL METHODS

N. T3 Uptake in Serum
Aria HT

1. Principle

The reference or sample is incubated in the incubation tray with 125-I labeled T3. Binding equilibrium is established between the T3 and TBG in the serum. This mixture is then aspirated and injected over a solid-phase absorbent, and the unbound T3 binds to the absorbent. The T3 bound to serum-binding proteins does not attach to the solid-phase absorbent but flows into the free detector and is counted by the gamma detector as the "free T3." An eluting agent then passes over the solid-phase absorbent releasing the bound T3, which is counted as the bound fraction in the appropriate detector. The absorbent is then rinsed and made ready to receive the next sample.

On the Aria HT system, all critical measurements of volume and timing, separation of free and bound fractions, radioactivity counting, and data reduction are done without operator intervention.

T3 uptake determinations are particularly useful in assessing (1) increases in TBG concentration in women who are pregnant or taking estrogens for contraception or (2) decreases, in nephrotic individuals. Total T4 in serum increases or decreases with similar changes in TBG levels in normal persons. Thus, the T3 uptake aids in interpreting total T4 values. The Aria HT T3 uptake assay automates the determination.

2. Specimen

a. Use only particulate-free serum. Hemolyzed and lipemic samples should not be used.

b. Minimum sample size is 70 ul, optimum size is 120 ul or greater.

c. Make sure that participant samples contain no countable radioactivity since it may lead to erroneous results.

d. Before being analyzed, the samples may be stored, at 2-8 °C. If storage exceeds 24 hours, the sample should be frozen. If specimens are not to be used immediately, cover with Parafilm and place in refrigerator. Cap specimens containers when not in use.

3. Reagents, Supplies and Storage

For in vitro diagnostic use only:

a. T3 Uptake Separation Chamber, catalog no. 633418, contains a solid-phase absorbent. Store at room temperature in a vial containing absorption buffer 1. Reference binding of 21%-40% indicates a viable chamber. Replace when back pressure attributed to the separation chamber is greater than the level 1 alert (alert 48).

b. T3 Uptake Prefilter, catalog no. 634417, contains filtering material. Store at room temperature. Replace filter when back pressure exceeds the level 1 alert (alert 48).

c. T3 Uptake Reference Serum, catalog no. 624284, contains stabilized human serum of a known percent uptake value and up to 0.1% sodium azide as a preservative; 20 ml per vial. Store tightly capped at 2-8 °C. Refer to expiration date on vial. Do not use if turbid.

d. 125-I Triiodothyronine Tracer, catalog no. 609111, contains 10.0 microcuries (370 kilobecquerels) on date of calibration; 5

mls/vial. See decay chart for dilution instructions. Store tightly capped at 2-8 °C. Refer to expiration date on bottle. Do not use if turbid.

e. Sample Buffer 3, catalog no. 601811, consists of an aqueous solution containing phosphate, sodium chloride, BSA, and 0.02% sodium azide as a preservative (pH 7.4±0.1). Volume is 800 ml. Store at room temperature. Refer to expiration date on bottle. Do not use if turbid.

f. Absorption Buffer 1, catalog no. 601129, aqueous solution contains sodium phosphate, and 0.02% sodium azide as a preservative (pH 7.4±0.1). Volume is 2.0 L. Store at room temperature. Refer to expiration date on bottle.

g. Elution Buffer 7, catalog no. 627518, aqueous acetonitrile solution contains glycinate (pH 2.5±0.2). Volume is 2.0 L. Store at room temperature. Refer to expiration date on bottle.

h. Gamma cocktail, catalog no. 527119, contains an alkyl immidazolinium dicarboxylate salt. Weight is 28 g. For use, add concentrate to 3.8 L distilled water and mix well. Avoid contact with skin. Store at room temperature. Refer to expiration date on bottle for concentrate. For diluted reagent, use within 4 weeks.

   Note: Gamma cocktail must be mixed before being used.

i. Rinse Solution 2, catalog no. 607614, distilled water and preservatives; contains 0.02% sodium azide. Volume is 2.0 L. Store at room temperature. Refer to expiration date on bottle.

j. Sample Handler Rinse, catalog no. 636118. Distilled water and preservatives; contains 0.02% sodium azide. Volume is 2.0 L Store at room temperature. Refer to expiration date on bottle.

k. LiquiCap, catalog no. 642312, contains polydimethylsilozane. Volume is 35 ml. Store at room temperature. Stability: Refer to expiration date on bottle.

4. Calibration

No standards are used in this procedure. T3 uptake uses a reference serum sample.

5. Quality Control Material

Two levels of Lyphochek controls, levels I and III, will be run in duplicate, one level each at the beginning and end of each analytical run.

To reconstitute the product, with a class A volumetric pipet add 5.0 ml of type I water to the desired vials. Replace the rubber stopper and allow the reconstituted material to stand at least 15 minutes. After that time, invert the bottle several times to ensure that the contents are thoroughly mixed. Before initial use, inspect each vial carefully for undissolved material. Reconstituted controls should be stored at 2-8 °C and are stable for 7 days.

6. Procedure

a. All reagents and samples must be brought to room temperature before they are used, but to minimize deterioration, they should not be left at this temperature longer than necessary.

b. Dilution of 125-I T3 tracer. The chart shows 125-I T3 tracer dilution requirements for T3 uptake with 100 cycles and days after calibration. The 125-I T3 tracer is supplied as a 5 ml volume containing 10 uCi along with the date of calibration. Using this information, dispense the amount of tracer into a 30-ml amber bottle (catalog no. 64). The required amount of tracer diluent (sample buffer 3) is then added to the bottle. Store the diluted tracer at 2-8 °C with the nonperforated cap in place. Diluted tracer should be used within 24 hours.

For example: The calibration date is January 4th, and the date of dilution is January 25. There are 21 days from calibration to the use date. For 100 cycles, the table indicates that 1.9 ml of tracer and 13.1 ml of sample buffer 3 (diluent) should be mixed.

125-I T3 Uptake Dilution Chart for 100 Cycles in Two Runs

No. days after calibration	Tracer	Diluent Sample Buffer 3
1	1.5	13.5
5	1.6	13.4
11	1.7	13.3
15	1.9	13.1
21	1.9	13.1
25	2.0	13.0
31	2.1	12.9
35	2.2	12.8
41	2.4	12.6
45	2.5	12.5
51	2.8	12.2
61	3.0	12.0
Final Volume	15.0 ml	

c. Pipet Standards, Controls, and Participant Preparation:

(1) Reference Preparation:

Volume of reference material dispensed depends on the number of samples to be assayed. The Aria HT will restandardize itself as programmed by the operator. The chamber binding has been demonstrated stable, ±3%, up to 100 cycles. Each standardization consists of the duplicate assay of the reference from a single cup:

Volume to Dispense	No. of Aspirations
At least 120 ul	2
At least 220 ul	4
At least 320 ul	6

Add the required amount of reference to one sample cup.

(2) Preparation of Controls:

One to three controls can be run on the Aria HT. The volume of control serum required is determined by the number of times each control is to be assayed and the operator enters in the test run parameters. Each control can be assayed several times from the same sample cup.

Volume to Dispense	No. of Aspirations
At least 70 ul	1
At least 120 ul	2
At least 170 ul	3, etc.

Add the appropriate amount of each control to separate sample cups. Overlay the dispensed controls with 50 ul of LiquiCap. Ensure dispersion of the LiquiCap by tipping the tray.

(3) Preparation of participant samples:

If samples have been frozen, thaw, mix, and recentrifuge at 1,000 X g for 5 minutes (2-25 °C). Dispense reference samples in sufficient volume for the aspirations programmed (320 ul for six warm-ups is recommended) into the first cup of the unknown samples. Add a minimum of 70 ul of each participant's serum (120 ul for duplicate assay from the same cup) to subsequent sample cups. Overlay the dispensed samples with 50 ul of LiquiCap. To make sure that the LiquiCap is dispersed, tip the tray.

d. For instrument set-up instructions, see Aria HT Instruction Manual.

7. Calculations

All samples are assayed, and the binding obtained is compared with that for the previously run reference and calculated as described below.

Description of Data Reduction Method

$$\% \text{ Recovery} = \frac{F(s) + B(s)}{\text{Total (R)}} \times 100$$

$$\% \text{ B(R)} = \frac{B(R)}{F(R) + B(R)} \times 100$$

$$\% \text{ B(s)} = \frac{B(s)}{F(s) + B(s)} \times 100$$

$$\text{T3 Uptake(s)} = \frac{(\%B(s))\ (\%UP(R))}{\%B(R)}$$

$$\text{T3 Ratio(s)} = \frac{\text{T3 Uptake(s)}}{30}$$

Where: F(R) = Free T3* not bound to chamber of the reference serum

B(R) = Bound T3* eluted from chamber of the reference serum
F(s) = Free T3* not bound to chamber of the unknown sample
B(s) = Free T3* eluted from chamber of the unknown sample
Total (R) = Mean total counts of reference samples
%UP(R) = Calibration value for the reference serum provided on the label.

8. Reference

Becton Dickinson. Instruction manual for determination of T3 uptake using the Aria HT. Orangeburg, New York: Becton Dickinson, 1982.

V. ANALYTICAL METHODS

O. Thyroid Stimulating Hormone (hTSH) in Serum
Aria-HT

1. Principle

The Becton Dickinson Immunodiagnostics Recyclable Immunoradiometric Assay (IRMA) for hTSH in which the Aria HT concept is used provides a sensitive, precise, and immunochemically specific assay for hTSH levels of clinical significance.

In the Becton Dickinson IRMA for hTSH, as carried out by the Aria HT system, the hTSH in the sample (standard or serum sample from a participant) is automatically mixed with a limiting amount of 125-I anti-hTSH, and the mixture is incubated for 1 hour at 37 °C. The concentration of 125-I anti-hTSH is sufficient to convert the hTSH to a radioactive complex, but does not saturate the antibody binding sites on the hTSH present in the radiolabeled complex. Thus, when the program of the Aria HT allows the reaction mixture to flow through the solid support antibody chamber containing anti-hTSH, the hTSH in the immune complex binds to the immobilized anti-hTSH to form a "sandwich." Unreacted (free) 125-I anti-hTSH is washed through the solid support antibody chamber and passes into the free detector, where it is counted as the "free" fraction. During the elution setup, the immune complex is then counted as the "bound" fraction in the bound detector. The percent of the total 125-I radioactivity in the bound fraction is directly proportional to the concentration of hTSH in the standard or participant sample.

On the Aria HT system, all critical measurements of volume and timing, all reagent additions to the incubation mixture, the separation of free and bound fractions, radioactivity counting, and data reduction are done without operator intervention.

Human thyrotropin (thyroid stimulating hormone, hTSH) is a protein hormone secreted by specialized cells, the thyrotrophs, in the anterior of the pituitary gland. The physiological function of this hormone is to stimulate the pathway for iodine metabolism in the thyroid: uptake of iodide from the blood, conversion of iodide to the thyroid hormones, and release of the hormones from the thyroid.

The secretion of hTSH from the pituitary gland is controlled by the hypothalamus, and this control is exerted via a peptide hormone, thyrotropin releasing hormone (TRH). The release of TRH is, in turn, regulated by signals that arise in the thalamus and cortex. TRH, isolated from sheep and hogs, is a tripeptide amide. This hormone reaches the pituitary by a system of blood vessels referred to as the hypothalamoenhypophysial portal system. The releasing factor appears to stimulate the membrane-bound adenylate cyclase of

the thyrotrophs, thereby increasing both the rate of synthesis and the release of hTSH. Thyroxine and triiodothyronine interfere with the release of hTSH by TRH. Thus, the secretion of hTSH is under negative feedback control by the thyroid hormones. If the level of the circulating thyroid hormone is low, secretion of thyrotropin increases. Conversely, when thyroid hormone secretion increases, hTSH secretion is inhibited. These complex interrelationships demonstrate the necessity of accurate hTSH determinations in the evaluation of clinical disorders of pituitary-thyroid function. Hence, this procedure is useful as an ancillary aid in the diagnosis of hypothyroidism.

2. Specimen

   a. Use only particulate-free serum. If serum is hemolyzed, do not use it. Plasma from blood anticoagulated in EDTA or heparin is also acceptable.

   b. Minimum sample size is 240 ul; optimum size is 280 ul or more.

   c. Make certain that clinical samples contain no exogenous gamma-emitting radioactivity, it may lead to erroneous results.

   d. If specimens are not run immediately, they may be stored at 2-8 ^{o}C. If not run within 24 hours, specimens are to be stored at -20 ^{o}C. Mix well upon thawing. Do not use if specimen has become turbid. Cap specimen containers when not in use.

3. Reagents, Supplies and Storage

   For in vitro diagnostic use.

   a. HTSH Antibody Chamber, catalog no. 621676, contains hTSH antibody covalently bound to a solid support. Refrigerate at 2-8 ^{o}C in a vial containing about 10 ml adsorption buffer 7. The percent binding difference between the 0 and 64 uIU/ml standards should be greater than 23%. Replace when back pressure attributed to the antibody chamber is greater than the level 1 alert (alert 48).

   b. hTSH Prefilter, catalog no. 621677. Store at room temperature. Replace prefilter when back pressure exceeds the level 1 alert (alert 48).

   c. hTSH Standards, catalog no. 634816, contains hTSH in stabilized human plasma with preservatives, including 0.1% sodium azide.

Standard	hTSH Concentration in ulU/ml	Quantity in ml	No. of Vials
Zero	0	8	2
A	1	4	1
B	2	4	1
C	4	4	1
D	8	4	1
E	16	4	1
F	32	4	1
G	64	8	1

Store at 2-8 °C. Refer to expiration date on bottle.

d. 125-I hTSH antibody, catalog no. 622176, contains 125-I hTSH antibody in TRIS buffer with sodium chloride (NaCl), hydrochloric acid (HCl), ethylenediamine tetraacetic acid (EDTA), bovine serum albumin (BSA), normal rabbit serum (NRS), and preservative. Each bottle contains 5 microcuries (uCi) (185 kilobecquerels) on date of calibration. Volume is 8 ml.

e. Absorption buffer 7, catalog no. 621978, aqueous solution containing TRIS, bovine serum albumin, sodium chloride and preservative, pH 8.0±0.01. Volume is 2.0 L. Store at room temperature. Refer to expiration date on bottle.

f. Elution buffer 6, catalog no. 622079, aqueous glycinate buffer containing preservative, pH 2.0±0.1. Volume is 2.0 L. Store at room temperature. Refer to expiration date on bottle.

g. Gamma cocktail, catalog no. 627119, contains an alkyl immidazolinium dicarboxylate salt. Minimum weight is 38 g. For use, add concentrate to 3.8 L of distilled water and mix well. Avoid contact with skin. Store at room temperature. Refer to expiration date on bottle for concentrate. For diluted reagent, use within 4 weeks.

Note: Gamma cocktail must be mixed before being used.

h. Rinse solution 2, catalog no. 607614, distilled water, and preservatives; contains 0.02% sodium azide. Volume is 2.0 L. Store at room temperature. Refer to expiration date on bottle.

i. Sample buffer 9, catalog no. 640514, aqueous solution contains TRIS, bovine serum albumin, sodium chloride, and preservative, pH 8.0±0.1. Volume is 2.0 L. Store at room temperature. Refer to expiration date on bottle.

j. Sample handler rinse, catalog no. 636118, distilled water and preservatives; contains 0.02% sodium azide. Volume is 2.0 L. Store at room temperature. Refer to expiration date on bottle

k. LiquiCap, catalog no. 642312, contains polydimethylsiloxane. Volume is 35 ml. Store at room temperature. Refer to expiration date on bottle.

4. Calibration

a. The volume of standard dispensed depends on the number of samples to be assayed. The Aria HT will restandardize as programmed by the operator. The chamber binding has been demonstrated stable ±3%., up to 60 cycles.

Each restandardization is performed from the same sample cups.

Volume to Dispense	No. of Aspirations
At least 240 ul	1
At least 480 ul	2
At least 720 ul	3, etc.

b. Add the required amount of standard to each of the following sample cups.

Standard	Cup
0 to ulU/ml	1
1.0 ulU/ml	2
2.0 ulU/ml	3
4.0 ulU/ml	4
8.0 ulU/ml	5
16.0 ulU/ml	6
32.0 ulU/ml	7
64.0 ulU/ml	8

5. Quality Control Material

Two levels of Lyphochek controls, levels II and III, are run in duplicate, one level each at the beginning and end of each analytical run.

To reconstitute the product, add 5.0 ml of type I water with a class A volumetric pipet to the desired vials. Replace the rubber stopper and allow the reconstituted material to stand at least 15 minutes. After that time, invert the bottle several times to ensure that the contents are thoroughly mixed. Inspect each vial carefully for undissolved material before initial use. Reconstituted controls should be stored at 2-8 °C and are stable for 7 days.

(One to three controls can be run on the Aria HT. The volume of control serum required is determined by the number of times each control is to be assayed as entered by the operator in the test run parameters. Each control can be assayed four times from the sample cup. An additional cup must be used if more aspirations will be required.)

6. Procedure

a. All reagents and samples must be brought to room temperature before use, but should not be left at this temperature once it has been reached.

b. Standard curve preparation (see Section 4, Calibration).

Overlay dispensed standards with 50 ul LiquiCap. To make sure that the LiquiCap is dispersed, tip the tray.

c. Preparation of controls: To run each control in duplicate, add the appropriate amount of each control to separate cups.

Overlay the dispensed controls with 50 ul LiquiCap. To make sure that the LiquiCap is dispersed, tip the tray.

d. Preparation of participant samples: If samples have been frozen, thaw, mix and recentrifuge after thawing at 1,000 X g for 5 minutes (2 to 8 °C).

e. Dispense zero standard in sufficient volume for the aspirations programmed (480 ul for two warm-ups is recommended) into the first cup of the unknowns. This is the warm-up position.

f. Add a minimum of 240 ul of each participant serum (480 ul for duplicate assay from the same cup) to subsequent sample cups.

g. Overlay the dispensed samples with 50 ul LiquiCap. To make sure that the LiquiCap is dispersed, tip the tray.

h. For instrument set-up instructions, see Aria HT Instruction Manual.

7. Calculations

Using an iterative technique, the HT computer determines certain standard curve parameters which are used in determining the concentrations in samples.

8. Reference

Becton Dickinson. Instruction manual for determination of hTSH using the Aria HT. Orangeburg, New York: Becton Dickinson, 1983.