Laboratory Procedures

Used by the Division of Environmental Health Laboratory Sciences, Center for Environmental Health, Centers for Disease Control

For the Hispanic Health and Nutrition Examination Survey (HHANES) 1982-1984

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
CENTERS FOR DISEASE CONTROL
ATLANTA, GEORGIA 30333

Laboratory Procedures Used by the Division of Environmental Health Laboratory Sciences Center for Environmental Health, Centers for Disease Control for the Hispanic Health and Nutrition Examination Survey (HHANES) 1982-1984

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CONTENTS

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I. Introduction

This manual documents the the biochemistry portion of the Hispanic Health and Nutrition Examination Survey (HHANES) performed at the Centers for Disease Control. It is a laboratory workers' manual describing nutritional biochemistry analyses as they were performed in a field survey from 1982 through 1984.

Sections II and III describe the procedures used by the field laboratory staff members (who are part of the Health Examination Field Operations Branch, Division of Data Services, National Center for Health Statistics (NCHS)) in collecting and processing specimens from examinees for the laboratory analyses in HHANES. Analytical methods used by the Division of Environmental Health Laboratory Sciences, Center for Environmental Health (CEH), Centers for Disease Control (CDC), are fully described in Section IV, and Section V presents the quality control data from these analyses.

The Metabolic Biochemistry Branch, Division of Environmental Health Laboratory Sciences, CEH, CDC, served as the coordinating laboratory for biochemistry analyses and data processing for HHANES. In addition, the Nutritional Biochemistry Branch researched, developed, and validated some of the clinical and nutritional biochemistry methods for use in this survey and for future implementation.

Serum, plasma, and whole-blood specimens collected from survey participants by the NCHS field laboratory personnel were sent to CDC from 30 field locations. These specimens were inventoried and distributed for analyses to CDC laboratories and to several outside laboratories. At CDC, pertinent identification data and all biochemical and hematological data on 11,310 survey participants were entered and maintained in a master computer file; periodic updates and a final, complete master tape record were furnished to NCHS for future distribution by the National Technical Information Service (NTIS), along with other NCHS-HHANES data tapes.

Many of the biochemistry analyses for HHANES were performed by the Division of Environmental Health Laboratory Sciences. Analyses for protoporphyrin, iron, total iron-binding capacity, blood lead, serum and red cell folate, vitamins A and E, and ferritin were performed in the HANES Laboratory Section, Metabolic Biochemistry Branch. Glucose levels were measured in the Clinical Trials Section, Metabolic Biochemistry Branch. The methods used were investigated before the start of HHANES and were validated in studies that confirmed their analytical soundness. Before required changes in methods were made, extensive comparison studies were performed. In addition, biological control materials were developed for use in assuring the quality and comparability of the data generated over the 3 years of the survey.

With the Hematology Branch, Host Factors Division, Center for Infectious Diseases, CDC, the Metabolic Biochemistry Branch provided training for the field laboratory personnel, developed procedures for sample collection and processing, produced a laboratory manual of these procedures, and—throughout the survey—periodically visited the field sites for the purpose of training new personnel and ensuring that proper techniques were being used, working with the supervisory lab technician.

In this manual, the use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

II. Field Specimen Collection

A. Venipuncture Blood Collection

Collect the following evacuated specimen tubes (Becton-Dickinson Co., Rutherford, NJ) from each age group:

Age Group (Years)	A (4-5)	B (6-11)	C (12-19)	D (20-74)	E (20-74) Glucose Tolerance
Label Color Code	Red	Green	Yellow	Blue	Orange
Tube Type:					
7-mL lavender-top (0.048 mL 15% K_3EDTA) B-D #6450	1	1	1	1	1
15-mL red-top B-D #6432	1	2	2	3	3
10-mL red-top B-D #6430				1	
15-mL blue-top (trace metal) B-D #A3218-XF974			1	1	
4-mL gray-top (8 mg potassium oxalate + 10 mg sodium fluoride) B-D #6445					3

B. Capillary Blood Collection

From all children less than 4 years old and from all other children for whom venipuncture is unsuccessful, collect the following samples by fingerstick, after scrubbing the fingertip for 20 sec with surgical soap ("SEPPS" antiseptic applicator, green soap tincture, No. 260071, Marion Scientific Co., Rockford, IL), wiping the fingertip with an alcohol pad, and drying the finger with a gauze square:

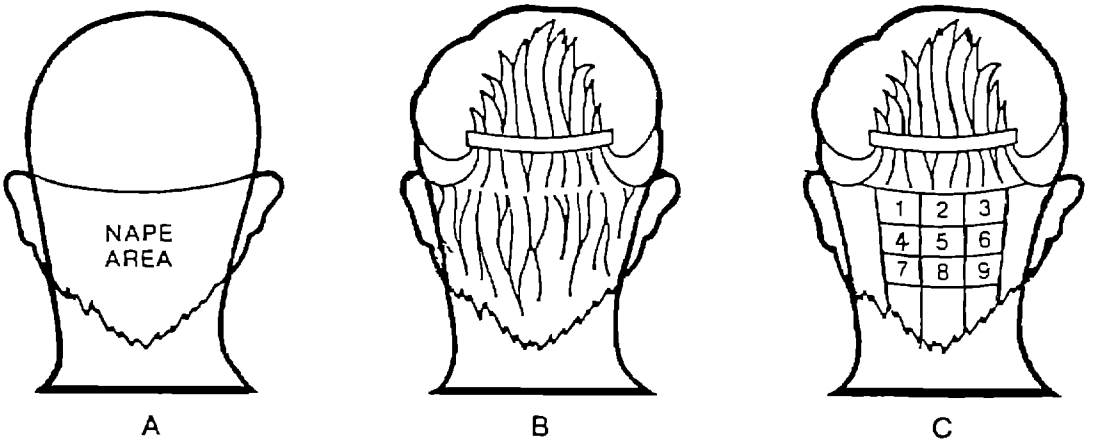
1. One 100-μL capillary tube (ESA EDTA-treated capillary sets, Environmental Science Associates, Bedford, MA) for blood lead determination).
2. Two 40-μL capillary tubes (for white blood cell, red blood cell, and hemoglobin determinations).
3. Two heparinized hematocrit tubes (for hematocrit determinations).
4. One 100-μL capillary tube for (erythrocyte protoporphyrin [EP] determination).
5. Two blood smears on glass slides for peripheral smear hematology evaluations).

C. Hair Collection

Administer hair-treatment questionnaires to all persons in age groups C and E, and collect hair samples from them according to the following protocol:

1. Use stainless steel surgical scissors, nylon combs, and aluminum clips; store these items in zip-lock plastic bags when not in use.
2. Disinfect the scissors, combs, and clips after each use.
   a. Dip the scissors, combs, and clips into isopropyl alcohol.
   b. Rinse them with distilled water.
   c. Rinse again with isopropyl alcohol from a polyethylene squeeze bottle.
   d. Dry in a dust-free environment (zip-lock bag).

3. Use disposable, powder-free plastic gloves when handling the hair specimens.
4. Collect the hair samples as follows:



A B C

   a. Collect samples from the nape area (diagram A).
   b. With a clean nylon comb, partition the hair between the ears (diagram B).
   c. Fasten the hair above the ears and out of the way with aluminum clips (diagram B).
   d. From 8-10 sites at the nape area (diagram C), gather 15-20 strands of hair. Hold the end of the hair, and cut the hair as close to the scalp as possible. A minimum of 200 mg is needed for analysis.
   e. From each cutting, cut off the 2 inches of hair that had been closest to the scalp, and store this sample in a zip-lock plastic bag.
   f. Discard the remaining hair.
   g. Seal the zip-lock bag.
   h. Affix the appropriate examinee identification (ID) number to the zip-lock bag.
5. Hair samples may be shipped with the other specimens to CDC. Place the hair samples on top of the other items in the shipper so that the bags do not get wet. Note hair collection on the Deck 511 (patient history) worksheet.

III. Field Specimen Processing

A. Specimen Separation

1. Centrifugation

Do all processing under the laminar-flow hood, using only materials that CDC has prescreened for contamination levels of trace metals. Allow blood in each red- or blue-top tube to clot for 30-40 min at room temperature. For red-top tubes, remove the stopper, loosen the clot from the sides of the tube with a clean, wooden applicator stick, and replace stopper. Do not open blue-top tubes until after centrifugation, to prevent contamination. Centrifuge and separate plasma from gray-top tubes as soon as possible. Place all tubes in centrifuge carriers; balance and centrifuge at 2400 rpm (RCF = 1115)[a] for 10 min.

2. Serum Separation and Pooling

Do not allow serum to remain in contact with the clot for longer than 1 h after the specimen is collected. Using a serum separator (Accusep serum separator # AL-51-0037, Acculab, Division of Precision Technology, Norwood, NJ), carefully remove the serum from the blue-top tube and pour it into the trace metals vial. Remove serum from all red-top tubes with serum separators and pool it into a 50-mL centrifuge tube, being careful to avoid introducing any cellular debris. If the serum from any red-top tube is grossly hemolyzed (for example, from traumatic venipuncture or mishandling), do not pool it with serum from the remaining tubes. If all the serum from an examinee is turbid, lipemic, or icteric, pool it and allocate as usual. (Not all biochemical tests may be performed on these specimens.) Stopper the 50-mL tube and mix its contents by inversion. Aliquot immediately or refrigerate at 4°C no longer than 4 h. Label each specimen as it is processed. If a patient is known to be hepatitis positive, write that information on the tube label and on the 511 worksheet.

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[a]Relative centrifugal force (RCF) = 0.0001118 X r X N^2, where r = 16.41 cm for the radius of the HL-4 rotor of a Sorvall GLC-1 centrifuge and N = 2400 rpm.

B. Specimen Allocation

Label all vials with the examinee's ID number and allocate specimens as follows:

1. Serum

Using clear serum only, fill as many vials as possible in order of priority shown in Table 1. If the serum from the blue-top tube is grossly hemolyzed, do not send it for analysis, but note "hemolysis" under "comments" on the 511 worksheet.

2. Plasma

Using a clean Pasteur pipette, remove plasma from each timed glucose tolerance test specimen after centrifugation and place in the appropriately labeled vial.

3. Whole Blood

Thoroughly mix contents in lavender-top (EDTA) tube; aliquot for blood lead specimen first, to minimize contamination. Restopper the lavender-top tube and mix the remaining contents again; perform hematological determinations. Prepare the hemolysate for red cell folate; aliquot the remaining whole blood for EP and carboxyhemoglobin specimens.

C. Protocols

See Tables 1 through 3.

TABLE 1.
HISPANIC HANES (HHANES) PROCESSING PROTOCOL FOR BIOCHEMISTRY SPECIMENS
(Listed in Order of Priority After Hematology Determinations)

Test ID Number	Test Name	Age Group(s)*	Sample Size (mL)	Specimen Type†	Collection Type	Vial Type§	Analyzed By¶	Other Remarks
1	Protoporphyrin	A,B,C,D,E	1	WB	7-mL EDTA lavender	P	CDC	100-μL if fingerstick on A or B
2	RBC folate	A,B,C,D,E	0.5	WB-HEM 1:5 dilution with ascorbic acid	7-mL EDTA lavender	P	CDC	500-μL SMI F pipette** 2000-μL SMI H pipette
3	Lead	A,B,C,D,E	1	WB lavender	7-mL EDTA	P	CDC	Aliquot first from EDTA tube, before CBC
4	Carboxy-hemoglobin	B,C,D,E	1	WB	7-mL EDTA lavender	SV	UP	Subsample†† Do not freeze
5a,b,c	Glucose tolerance	E	1,1,1	Pl	4-mL NaF gray	P	CDC	2-h test§§
6	Iron/total iron-binding capacity	A,B,C,D,E	3	S	10/15-mL red	P	CDC	
7	Folate	A,B,C,D,E	2	S	10/15-mL red	P	CDC	
8	Thiocyanate	B,C,D,E	1	S	10/15-mL red	SV	UP	Subsample†† Do not freeze
9	Vitamin A/E	A,B,C,D,E	1	S	10/15-mL red	P	CDC	
10	Cholesterol/ triglycerides	D,E	3	S	10/15-mL red	F	JHUH	Must be in clear tube for lipemia to be detected
11	Biochemistry Profile	D,E	2	S	10/15-mL red	P	NMSU	
12	Syphilis serology	C,D,E	1	S	10/15-mL red	P	CDC	
13	Pesticides (organic)	C,D	5	S	10/15-mL red	EPA—glass	EPA	Urine sample to be collected (subsample)¶¶
14	Trace Metals	C,E	5-7	S	15-mL blue	P	CDC	Specimens must be free of trace metal contamination***
15	Tetanus	A,B	1	S	10/15-mL red	P	CDC	
16	Ferritin	A,B,C,D,E	1	S	10/15-mL red	P	CDC	
17	Reserve	A,B,C,D,E	2	S	10/15-mL red	P	CDC	
18	Excess	C,D,E	1	S	10/15-mL red	P	CDC	
19	Excess	C,D,E	1	S	10/15-mL red	P	CDC	
White label	Trace Metals	C,E	—	Hair	—	Plastic bag	CDC	Subset***

*See Section IIA.

†Specimen Type:
- WB = Whole blood
- WB-HEM = Whole blood hemolysate for RBC folate analysis. 500 μL of EDTA-whole blood is added to 2.0 mL of 1-g/dL ascorbic acid, then mixed and frozen before shipping.
- Pl = Plasma
- S = Serum

§Vial Type:
- P = "Mini-vial" plastic scintillation vial, 6 mL, #6000169, Packard Instruments Corp., Downers Grove, IL (used for all samples unless otherwise noted)
- SV = Special glass vial with rubber septum
- F = Falcon 2027 disposable polystyrene tube, Becton-Dickinson Co., Oxnard, CA
- EPA = 20-mL glass jar supplied by the Environmental Protection Agency to prevent contamination of urine pesticide analysis, or 2-dram glass vial for serum samples

[¶]Analyzed by:

CDC = Centers for Disease Control
UP = University of Pittsburgh
JHUH = Johns Hopkins University Hospital
NMSU = New Mexico State University
EPA = Environmental Protection Agency, Bay St Louis, MS

[**]Scientific Manufacturing Industries, Emeryville, CA

[††]Carboxyhemoglobin and thiocyanate analyses are performed on specimens from all survey participants 6 years of age and older with odd-numbered identification numbers (ID's). This assay was discontinued after the first year of HHANES.

[§§]2-h glucose tolerance test is performed. A blood specimen is collected from a fasting participant. He/she is given Glucola® (#2604, Ames Diagnostics, Elkhart, IN), a 75-g carbohydrate-load cola solution. Additional specimens are collected 1 and 2 h after cola ingestion

[¶¶]Pesticide analyses are performed on specimens from all persons with even-numbered ID's from Group C and both odd and even numbered ID's from Group D. A urine specimen is also collected from these persons.

[***]Selected trace metal determinations will be performed on a subset of a field test of the HHANES specimens after the hair questionnaire data have been evaluated.

TABLE 2.
OVERALL ANALYTICAL PROTOCOL FOR HISPANIC HANES SPECIMENS

Age Group (Years of Age) Label Color	A (4-5)* Red	B (6-11) Green	C (12-19) Yellow	D (20-74) Blue	E (20-74) Orange
			WHOLE BLOOD		
	CBC Lead Protoporphyrin Red cell folate[†]	CBC Lead Carboxyhemoglobin Protoporphyrin Red cell folate[†]	CBC Lead Carboxyhemoglobin Protoporphyrin Red cell folate[†]	CBC Lead Carboxyhemoglobin Protoporphyrin Red cell folate[†]	CBC Lead Carboxyhemoglobin Protoporphyrin Red cell folate[†]
			SERUM		
	Ferritin Iron Total Iron Binding Capacity (TIBC) Folate[†] Vitamin A/E Tetanus	Ferritin Iron TIBC Folate[†] Vitamin A/E Thiocyanate Tetanus	Ferritin Pesticides[§] Syphilis Iron TIBC Folate[†] Vitamin A/E Thiocyanate Trace Metals[¶]	Ferritin Cholesterol Triglycerides Pesticides[§] Profile Syphilis Iron TIBC Folate[†] Vitamin A/E Thiocyanate	Ferritin Cholesterol Triglycerides Profile Syphilis Iron TIBC Folate[†] Vitamin A/E Thiocyanate Trace Metals[¶]
			PLASMA		
					Glucose tolerance[**]

*Children 6 months to 3 years old had protoporphyrin and lead analyses on blood collected by fingerstick technique.

[†]Special hematological subset only (See Table 3 for an explanation of the subset criteria.)

[§]Participants with even-numbered identifications only.

[¶]Selected trace metal determinations will be performed on a subset of the samples collected.

[**]2-h glucose tolerance tests are performed on this age group. A blood specimen is collected from a fasting participant. He/she is given Glucola® (#2604, Ames Diagnostics, Elkhart, IN), a 75-g carbohydrate-load cola solution Additional specimens are collected 1 and 2 h after cola ingestion.

TABLE 3.
CRITERIA FOR INCLUSION INTO THE SPECIAL HEMATOLOGICAL SUBSET*

Assessment/by Sex and Age	Value Less Than	Value Greater Than
White cell count (all)	3.5 (X 10^3)	13.0 (X 10^3)
Red cell count		
Males > 15 yrs	4.0 (X 10^6)	6.0 (X 10^6)
Females > 15 yrs, Children†	3.8 (X 10^6)	6.0 (X 10^6)
Hemoglobin		
Males > 15 yrs	13.5 g/dL	18.5 g/dL
Females > 15 yrs	11.0 g/dL	16.5 g/dL
Children†	11.0 g/dL	—
Hematocrit		
Males > 15 yrs	38.0%	56.0%
Females > 15 yrs	32.0%	50.0%
Children†	31.0%	—
Mean corpuscular volume		
Males > 15 yrs, Females > 15 yrs	79.5 μ^3	105.0 μ^3
Children†	74.5 μ^3	100.0 μ^3

*On the basis of these hematological indices, a participant was selected for the special hematological subset; serum and red cell folate analyses and differential smear analyses were performed. A control group was composed of "normal" participants whose identification numbers ended in 8. In addition, all women 18-44 years old were included.
†Persons under 16.

D. Shipping Procedures

Shipping instructions are fully discussed in *Hispanic HANES Examination Staff Procedures Manual for the Health and Nutrition Examination Survey, 1982-1984* (*1*).

1. CDC Shipments

All processed specimens from one survey participant that are to be shipped to CDC are placed in a sealable plastic bag and frozen upright. Shipments by express mail are made weekly in a large shipper containing the frozen samples, 12 lb dry ice cake, and the accompanying Deck 511 worksheet originals. Differential smears are collected in a slide box and are shipped at the end of a stand.

Vials identified as follows are shipped to CDC: 1, 2, 3, 5a, 5b, 5c, 6, 7, 9, 12, 14, 15, 16, 17, 18, and 19.

2. Other Shipments

a. Carboxyhemoglobin and Thiocyanate
Vials Nos. 4 and 8 are accumulated in the refrigerator and shipped weekly in a small styrofoam shipper with a coolant and a copy of the Deck 515 worksheet to: Department of Epidemiology, University of Pittsburgh, Pittsburgh, PA.

b. Biochemistry Profile
No. 11 vials are accumulated in the freezer and shipped weekly in a styrofoam shipper with dry ice and a copy of the Deck 512 worksheet to: PRI Clinical Services Laboratory, Holloman Air Force Base, NM.

Section III D Reference

1. National Center for Health Statistics. Hispanic HANES Examination Staff Procedures Manual for the Health and Nutrition Examination Survey, 1982-1984. Hyattsville, Maryland: National Center for Health Statistics, 1985. part 15a, 8-4 - 8-11.

c. Cholesterol/Triglycerides
No. 10 clear plastic tubes are accumulated in the freezer and shipped weekly in special plastic shippers with dry ice and a copy of the Deck 513 worksheet to: Lipid Research Clinic, Johns Hopkins University Hospital, Baltimore, MD.

d. Pesticides
The special glass containers provided by the Environmental Protection Agency (vial 13, serum, and a sample-number-labeled vial with 20 mL of urine) are accumulated in the freezer and shipped weekly in a special metal shipper with dry ice and a copy of the Deck 510 worksheet to: U.S. Environmental Protection Agency, Toxicant Analysis, Bay St. Louis, MS.

E. Storage Procedures

Upon arrival at CDC, the specimens are sorted by vial type and are stored at -20°C.

IV. Analytical Methods

A. Erythrocyte Protoporphyrin

1. Principle

Free erythrocyte protoporphyrin (FEP) is measured by a modification of the method of Sassa et al. (*1*). Protoporphyrin is extracted from EDTA-whole blood into a 2:1 (v/v) mixture of ethyl acetate-acetic acid, then back-extracted into diluted hydrochloric acid. The protoporphyrin in the aqueous phase is measured fluorometrically at excitation and emission wavelengths of 404 and 655 nm, respectively. Calculations are based on a processed protoporphyrin IX (free acid) standard curve. The final concentration of protoporphyrin in a specimen is expressed as micrograms per deciliter of packed red blood cells (μg/dL RBC); a correction for the individual hematocrit is made.

2. Instrumentation

a. Perkin-Elmer model 650-10 spectrofluorometer, with R928 photomultiplier tube, xenon lamp, and custom-made microcell (10- X 75-mm) holder positioned to allow the passage of light through the aqueous phase only (Perkin-Elmer Corp., Norwalk, CT)
b. Model 56 recorder (Perkin-Elmer Corp.)
c. Cary model 119 double-beam spectrophotometer (Varian Associates, Palo Alto, CA)
or: Acta CII and CIV double-beam spectrophotometer (Beckman Instruments, Fullerton, CA)
d. Vortex mixer (Fisher Scientific Co., Fairlawn, NJ)
e. Micromedic APS-2 pipetting station with 20-μL sampling and 200-μL dispensing pumps and reagent dispenser, with inserts in racks modified to accept 10- X 75-mm tubes (Micromedic Systems, Division of Rohm and Haas, Horsham, PA)
f. Mettler model H18 analytical balance (Mettler Instrument Corp., Hightstown, NJ)
g. Beckman TJ-6 centrifuge (Beckman Instruments Co.)
h. Hamilton dispenser, with 2.5-mL syringes and dispensing block
(Hamilton Co., Reno, NV)
i. Micromedic high-speed automatic diluter with 1.0-mL dispensing pump (Micromedic Systems)

3. Materials

a. Protoporphyrin IX, dimethyl ester, 99.3% purity; grade 1 (Sigma Chemical Co., St. Louis, MO)
NOTE: Store at -20°C over a desiccant. Purchase of one lot is recommended.
b. Ethyl acetate, high-pressure liquid chromatography (HPLC) grade. (J.T. Baker Co., Phillipsburg, NJ)
c. Acetic acid, glacial, "Baker analyzed" (J.T. Baker Co.)
d. Hydrochloric acid, concentrated, "Baker Analyzed" (J.T. Baker Co.)
e. Kimble 10- X 75-mm disposable glass culture tubes
(Kimble Div., Owens-Illinois Co., Toledo, OH)
f. Parafilm M (American Can Co., Greenwich, CT)
g. Actinic glass volumetric flasks (Corning Glassworks, Corning, NY)
NOTE: All nondisposable glassware used in this assay should be washed in 10% (v/v) hydrochloric acid and rinsed six times with deionized water.
h. Formic acid, 88%, reagent grade (J.T. Baker Co.)
i. Deionized water, greater than or equal to 1.0 megaOhm-cm at 25°C (Continental Water Co., Atlanta, GA)
j. Sodium chloride (NaCl), ACS certified (Fisher Scientific Co., Fairlawn, NJ)

4. Reagent Preparation

a. *7.0 mol/L hydrochloric acid (HCl) (for hydrolysis)*
Dilute 551 mL concentrated HCl to volume with deionized water in a 1-L volumetric flask.
b. *1.62 mol/L HCl (for daily absorbance readings)*
Dilute 141 mL concentrated HCl to volume with deionized water in a 1-L volumetric flask.
c. *0.43 mol/L HCl (for analysis-extraction)*
Dilute 68 mL concentrated HCl to volume with deionized water in a 2-L volumetric flask.

d. *1.5 mol/L HCl (for blanking spectrophotometer)*
Dilute 118 mL concentrated HCl to volume with deionized water in a 1-L volumetric flask.
NOTE: These dilutions assume concentrated HCl to be 12.7 mol/L. The molar concentration of different lots of HCl should be calculated by using the following formula:

$$\text{mol/L} = \frac{\text{relative density X \% HCl}}{35.453}$$

e. *2:1 (v/v) ethyl acetate-acetic acid*
Working under a hood, combine 200 mL ethyl acetate and 100 mL glacial acetic acid. Mix the solution well; this volume is sufficient for the standards, controls, and 80 specimens. (Prepare this reagent daily, immediately before sampling the whole blood.)

f. *0.85 g/dL saline*
Dilute 8.5 g sodium chloride with deionized water to equal 1 L. Mix well. If desired, aliquot in 10-mL volumes in screw-capped glass tubes for convenience. (Store at 4°C.)

5. Standard Preparation

NOTE: Prepare all standard solutions in actinic glass volumetric flasks, in very reduced light.

a. *Protoporphyrin IX standards*
Concentrations are expressed in terms of protoporphyrin IX free acid after the dimethyl ester has been hydrolyzed. The millimolar absorptivity of protoporphyrin IX free acid has conventionally been determined in 1.5 mol/L HCl; thus, the daily absorbance reading of the hydrolysate is determined at this acid concentration (*2*).

(1) *20 mg/dL protoporphyrin IX free acid hydrolysate (stock standard)*
Measure 42.0 mg protoporphyrin IX dimethyl ester (PPIX DME). Dilute to volume in a 200-mL actinic volumetric flask with 7 mol/L HCl, washing PPIX off weighing paper with a few drops of formic acid. Add a small stirring bar, cover the flask with aluminum foil, and mix contents at 20-25°C for 3 h, using a magnetic stirrer. (Prepare weekly.)

(2) *1000 mg/dL intermediate stock*
After 3 h, dilute 25.0 mL of 20 mg/dL solution with deionized water to volume in a 500-mL actinic volumetric flask to yield a 1000 μg/dL solution, which is 0.35 mol/L with respect to HCl. (Prepare weekly.)

(3) *100 mg/dL standard for daily absorbance readings*
Dilute 10.0 mL of 1000 μg/dL intermediate stock to volume in a 100-mL actinic volumetric flask with 1.62 mol/L HCl to yield a 100 μg/dL protoporphyrin IX standard, which is 1.5 mol/L with respect to HCl. Use an aliquot of this standard for absorbance readings, as in section VI.B.

NOTE: The theoretical concentration of this solution with respect to protoporphyrin IX free acid (PPIX FA) is calculated as follows:

$$\text{(a)}\quad \frac{42\text{ mg PPIX DME}}{200\text{ mL}} \times \frac{562.27\text{ mg PPIX FA}}{590.72\text{ mg PPIX DME}} = .1999\text{ mg PPIX FA/mL}$$

$$\text{(b)}\quad \frac{.1999\text{ mg PPIX FA}}{\text{mL}} \times \frac{25\text{ mL}}{500\text{ mL}} \times \frac{10\text{ mL}}{100\text{ mL}} = .0009975\text{ mg/mL PPIX FA} \quad (99.75\ \mu\text{g/dl})$$

$$\text{(c)}\quad \frac{99.95\ \mu\text{g}}{1\text{ dL}} \times \frac{1\text{ mmol}}{562.27} \times \frac{10\text{ dL}}{1\text{ L}} \times \frac{1\text{ mg}}{1000\ \mu\text{g}} = .00178\text{ mmol/L PPIX FA}$$

(4) *100 mg/dL standard for dilutions*
Dilute 5.0 mL of 1000 μg/dL intermediate stock to volume with 0.43 mol/L HCl in a 50-mL actinic volumetric flask.

(5) *0-80 mg/dL working standards*
Prepare the following working standards daily by diluting the 100 μg/dL standard with 0.43 mol/L HCl according to the following dilution scheme, using a Micromedic APS-2

equipped with 50-μL sampling and 200-μL dispensing pumps, and the reagent dispenser.

NOTE: Be sure to work under very subdued lights when diluting and extracting the standard materials, since they are photo-labile.

Working Standard Concentration (μg/dL)	Volume 1000 μg/dL Standard (μL)	Volume 0.43 mol/L HC1 Diluent (μL)	Final Volume (μL)
80	400	4600	5000
70	350	4650	5000
60	300	4700	5000
50	250	4750	5000
40	200	4800	5000
30	150	4850	5000
20	100	4900	5000
10	50	4950	5000
0	0	5000	5000

6. Procedure

To protect hands against acids and solvents during sampling, wear latex gloves. To avoid evaporation or degradation of specimens, process samples as rapidly as possible. After centrifugation, samples are stable for 1-3 h.

a. Thaw specimens and quality control materials of frozen EDTA-whole blood at room temperature.

   NOTE: Control pools with elevated levels of FEP are prepared from blood (EDTA-anticoagulated) collected from cows that have been fed lead acetate.

b. Using the spectrophotometer and quartz cuvettes, measure absorbance at wavelength-maximum (approximately 407-408 nm) of the 100 μg/dL in 1.5 mol/L HCl standard solution against a blank of 1.5 mol/L HCl, scanning from 380-420 nm. This measurement will be used in determining standard concentrations. Clean cuvettes with 5% Contrad detergent solution after use, and rinse them thoroughly with deionized water followed by ethanol to remove water droplets.

c. Prepare the working standard dilutions from 100 μg/dL standard in 0.43 mol/L HCl, using 0.43 mol/L HCl as a diluent. These dilutions are unstable; therefore, prepare them as rapidly as possible.

d. Prepare the 2:1 ethyl acetate-acetic acid mixture, and fill the dispenser bottle of the Micromedic high-speed dilutor for delivering 1.0 mL of reagent. Fill the dispenser bottle of the Hamilton dilutor with 0.43 mol/L HCl for delivery of 1.0 mL. (When using dilutors, place them under a hood to minimize fumes.)

e. Before sampling, vortex thoroughly each standard dilution, quality control pool, or whole blood specimen. Using the APS-2, transfer 10 μL of the sample to a 10- X 75-mm disposable glass tube, in duplicate.

f. Add 1.0 mL of the 2:1 ethyl acetate-acetic acid mixture to each sample. Mix thoroughly for 10 sec.

g. Add 1.0 mL of the 0.43 mol/L HCl to each sample. Wrap tube with Parafilm, and mix thoroughly for 10 sec.

h. Sample in this order: standards, quality control pools, and whole-blood specimens in duplicate.

i. Prepare four blank tubes (0 standards) with 1.0 mL each of ethyl acetate-acetic acid and 0.43 mol/L HCL, with 10 μl 0.43 HCL as sample.

j. When all sampling is completed, centrifuge all tubes for 4 min at 1400 rpm.

k. For samples outside the range of the standard curve, use a smaller sample size or dilute sample with 0.85 g/dL saline. For example,
   5 μL = 1:2 dilution
   2 μL = 1:5 dilution
   100 μL sample and 900 μL saline = 1:10 dilution, 10 μL sample used
l. Perkin-Elmer 650-10 spectrofluorometer settings:

Parameter	Setting
slit(s) width	10 nm
photomultiplier tube	R928 Hamamatsu
cuvettes	10- X 75-mm in microcell adapter
range	1
PM gain	normal
response	normal
mode	normal
scan	off
wavelengths	404 nm excitation
	655 nm emission

m. Allow 1 h for the 650-10 to warm up and stabilize after the xenon lamp has been ignited.
n. Following the instruction manual, zero the Model 56 recorder with "Recorder Zero" and "MEAS."
o. With shutter closed and sensitivity set on "1," zero the 650-10 spectrofluorometer by using "Zero Adjust" with "Zero Suppression" *OFF*.
p. Open shutter. Turn "Zero Suppression" on. Put tube with blank solution in sample compartment, and zero the digital readout carefully by using the zero suppression knob.
   NOTE: Because of tube-to-tube variance, be sure to check several blank tubes and take the average amount of the blank to be zeroed out.
q. Adjust sensitivity of fluorometer by reading 50 and 60 μg/dL PPIX standards and adjust "fine" sensitivity to read each value, respectively, as 50 and 60 fluorescent units. (Standard curve has a tendency to lose linearity on the 70 to 80 μg/dL standards by approximately 2 fluorescent units.)
r. Proceed to read the standard curve, quality control pools, and samples.

7. Calculations

The millimolar absorptivity of protoporphyrin IX free acid in 1.5 mol/L HCl has been determined in our laboratory to be 297 $\pm$ 1 (600 observations from 1976 to 1984). The purity of our material has been confirmed by elemental analysis and high-performance liquid chromatography of the extracted protoporphyrin IX free acid. Calculate the actual concentration of the 100 μg/dL (.00178 mmol/L) working standard, using the following equation:

$$A = \epsilon bc$$

Where:

A = absorbance reading
b = cuvette pathlength, 1 cm
c = concentration, in mmol/L
ϵ = millimolar absorptivity of protoporphyrin IX free acid in 1.5 mol/L HCl, 297

For example, if the daily absorbance reading of the 100 μg/dL standard at wavelength maximum is 0.520, then:

$$C = \frac{0.520}{(297\ \text{L/mmol-cm})\ (1\ \text{cm})} = .00175\ \text{mmol/L}.$$

Then: (.00175 mmol/L)(562.27 mg/mmol)(1000 μg/mg)(1L/10 dL)= 98.40 μg/dL PPIX FA

Consider 98.40 as a percentage of 100 μg/dL, and correct the standard curve accordingly:
10 μg/dL X 0.9840 = 9.84
20 μg/dL X 0.9840 = 19.68, etc.

Perform a linear regression, with x = corrected standard concentration and y = fluorescent intensity reading. Using the slope of the standard curve and assuming zero intercept, calculate the concentration of protoporphyrin IX per deciliter of whole blood for each specimen. To correct for hematocrit and express results as μg/dL of RBC, use this formula:

$$\frac{\mu g/dL \text{ whole blood}}{\text{hematocrit}} \times 100 = \mu g/dL \text{ RBC}$$

8. CDC Modifications

The following modifications of the original methods are based on CDC optimization experiments: (a) sample size increased from 2 μL to 10 μL; (b) ethyl acetate-acetic acid and 0.43 mol/L HCl volumes increased from 0.3 mL to 1.0 mL; (c) processed protoporphyrin IX standards used; (d) hydrolysis time for the dimethyl ester decreased from 48 h to 3 h, on the basis of the work of Culbreth et al. (*3*); and (e) 0.43 mol/L HCl was chosen as a diluent for maximum fluorescent intensity and stability of the extracted protoporphyrin IX.

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1. Sassa S, Granick JL, Granick S, Kappas A, Levere RD. Microanalyses of erythrocyte protoporphyrin levels by spectrophotometry in the detection of chronic lead intoxication in the subclinical range. Biochem Med 1973; 8:135-48.
2. Committee on Specifications and Criteria for Biochemical Compounds, National Research Council. Specifications and criteria for biochemical compounds. 3d ed. Washington, DC: National Academy of Science, 1972.
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B. Serum Iron and Total Iron-Binding Capacity

1. Principle

Serum iron and total iron-binding capacity (TIBC) are measured by a modification of the automated Technicon AAII-25 method, which is based on the procedures of Giovanniello et al. (*1*) and of Ramsey (*2*). Iron is quantitated by measuring the intensity of the violet complex formed in the reaction between ferrozine and Fe(II) in pH 4.7 acetate buffer at 562 nm. In TIBC tests, serum is mixed with a 400 μg/dL iron solution to saturate the iron-binding sites of the serum transferrin molecules. Magnesium carbonate is used to remove excess iron. Centrifugation is used to precipitate the magnesium carbonate, and the supernatant is then analyzed for iron.

2. Instrumentation

a. Technicon AutoAnalyzer I system (Technicon Instruments, Inc., Tarrytown, NY)
   (1) Sampler II or III with 30/h 2:1 cam
   (2) Pump II
   (3) Dialyzer—37°C, with two standard type C dialysis plates connected in series
   (4) Colorimeter—with 50-mm ID flowcell and 570-nm filters
   (5) Bristol recorder, with absorbance chart paper
   (6) Flow-rated tubing
b. Micromedic model 25000 automatic pipette, with 1.0-mL sampling and 5.0-mL dispensing pumps (Micromedic Systems, Division of Rohm and Haas, Horsham, PA)
c. Vortex mixer (Fisher Scientific Co., Fairlawn, NJ)
d. IEC centrifuge, Model UV (International Equipment Co., Needham Heights, MA)

3. Materials

a. 2.0-mL disposable conical bottom sample cups for AutoAnalyzers, HRI No. 8889-201409 (Lancer Division, Sherwood Medical Corp., St. Louis, MO)

b. Disposable filtering columns (Whale Scientific Co., Denver, CO)
c. "Ferrozine" iron reagent (3-(2 pyridyl)-5,6 bis (4-phenylsulfonic acid)-1, 2, 4, triazine, monosodium, monohydrate), 95% purity (Hach Chemical Co., Ames, IA)
d. L-ascorbic acid, 99.9% purity (J.T. Baker Co., Phillipsburg, NJ)
e. Magnesium carbonate (basic), Fisher certified (Fisher Scientific Co.)
f. Sodium hydroxide (NaOH), electrolytic pellets, ACS certified (Fisher Scientific Co.)
g. Sodium acetate, trihydrate, "Baker analyzed" (J.T. Baker Co.)
h. Hydrochloric acid (HCl), concentrated, reagent grade (J.T. Baker Co.)
i. Brij-35, 30% solution (Pierce Chemical Co., Rockford, IL)
j. 15- X 85-mm disposable culture tubes (Corning Glass Works, Corning, NJ), lot-tested for iron contamination
k. Iron wire, 99.9% purity (Mallinckrodt Chemical Works, St. Louis, MO)
l. Deionized water, greater than or equal to 1.0 megaOhm-cm at 25°C (Continental Water Co., Atlanta, GA)
m. Sodium chloride (NaCl), ACS certified (Fisher Scientific Co.)

4. Reagent Preparation

a. *0.2 mol/L hydrochloric acid with 3 g/dL sodium chloride*
To 250 mL of deionized water in a 2-L flask, add 34 mL of concentrated HCl, 60 g of NaCl, and 0.5 mL of Brij-35, 30% solution. Mix well and dilute to 2 L with water. (Prepare as needed; stable at 25°C.)
b. *1.0 g/dL ascorbic acid*
Add 8 g of L-ascorbic acid to 800 mL of 0.2 mol/L HCl with 3 g/dL NaCl. Mix well. (Prepare daily.)
c. *0.75 mol/L acetate buffer, pH 4.7 at 25° C*
Add 816.8 g of sodium acetate (f.wt. = 136.1) to 4 L of deionized water in an 8-L flask. Stir well, and dilute to volume with deionized water. Check to ensure that final pH is 4.7 ± 0.1, and adjust if necessary with 0.1 N NaOH or 0.1 N HCl. (Prepare weekly; stable at 25°C.)
d. *0.07 g/dL ferrozine*
Add 0.7 g ferrozine to 1 L of 0.75 mol/L acetate buffer solution and mix well. (Prepare weekly; stable at 25°C.)
e. *0.5 ml/L Brij-35 wash solution*
Add 1.0 mL Brij-35, 30% solution, to 2 L deionized water and mix well. (Prepare weekly.)
f. *0.1 mol/L hydrochloric acid (for standards)*
Add 8.3 mL concentrated HCl to 500 mL deionized water in a 1-L volumetric flask. Mix well and dilute to volume with water. Do not add Brij-35. (Approximately 5 L of this solution is required to prepare intermediate and working standards.)
g. *400 mg/dL iron saturating solution*
Dilute 2.0 mL of the 1.0 g/dL stock iron standard to volume in a 500-mL flask with deionized water. (Stable at 25°C.)
h. *1 mol/L NaOH (for cleaning)*
Dissolve 40 g sodium hydroxide pellets in 500 mL deionized water in a 1-L flask. Stir well, and dilute to volume. (Prepare as needed.)

5. Standard Preparation

a. *1.0 g/L stock iron standard solution*
Place 1.000 g iron wire in a 1-L volumetric flask. Add 12 mL of concentrated HCl and dissolve wire with slight warming. After wire is completely dissolved, cool flask to room temperature and dilute to volume with deionized water. (Stable indefinitely; store in a polyethylene container at 25°C.)
b. *50.0 mg/L iron intermediate stock solution*
Dilute 25 mL of the 1.0 g/L stock iron solution to 500 mL with 0.1 mol/L HCl. (Prepare each time new working standards are required.)
c. *Working iron standards*
In a series of 500-mL volumetric flasks, prepare dilutions from the intermediate standard as shown below. Dilute to 500 mL with 0.1 mol/HCl and mix well. (Prepare every 3 mo.)

WORKING IRON STANDARDS
(Dilute to 500 mL with 0.1 mol/L HCl)

mL of 50 mg/L Intermediate Standard	Final Concentration μg/dL Iron
3	30
5	50
8	80
10	100
15	150
20	200
25	250
30	300

6. Procedure

a. Preparation of serum samples for iron assay
Mix freshly drawn or thawed serum samples thoroughly, using a vortex mixer. Filter about 2 mL of serum into a 2.0-mL AutoAnalyzer sample cup, using a disposable plastic filtration column to remove fibrin.

b. Preparation of samples for TIBC assay
Using the Micromedic automatic pipette, add 0.8 mL of well-mixed filtered serum to 1.6 mL of 400 μg/dL iron saturating solution in 15- X 85-mm tubes. Mix well and allow tubes to stand for at least 15 min (at this point, if necessary, the samples may be tightly capped and kept at 4°C overnight). Add 0.2 g of basic magnesium carbonate directly to each tube of diluted serum. Mix contents of tubes; allow the tubes to stand for 45 min, mixing at 15-min intervals. Centrifuge the samples at 2500 rpm for 10 min to pack the magnesium carbonate. Decant the supernatant into 2.0-mL conical-bottom AutoAnalyzer sample cups, and proceed as with the iron analysis.

c. Quality control materials
Assay quality control pools in the same manner as samples, with this exception: TIBC cannot be accurately determined on reconstituted lyophilized serum.

d. Operation
Follow standard AutoAnalyzer protocol as described in *Practical Automation for the Clinical Laboratory* (*3*). Approximately 1.6 mL of serum is needed for each iron analysis and 1.6 mL of supernatant for each TIBC analysis. For every 40 specimens, standards and controls are analyzed in duplicate. Change flow-rated pump tubing and type C dialysis membranes every 4 days of analysis. Wash with 1N NaOH cleaning solution weekly to prevent protein buildup in colorimeter flowcell. For maximum sensitivity, the 300 μg/dL standard should read at least 0.450 absorbance units, making the use of scale expansion unnecessary.

7. Calculations

Calculate serum iron concentrations of specimens and diluted TIBC samples from the slope and y-intercept of the standard curve. Multiply the iron concentration of the diluted TIBC sample by the dilution factor of 3. Report serum iron and TIBC as micrograms of iron per deciliter of serum (μg/dL). Verify by dilution and/or reassay any serum iron concentrations less than 30 μg/dL or greater than 200 μg/dL and any TIBC concentrations less than 250 μg/dL or greater than 500 μg/dL. The analysis is linear to 1000 μg/dL.

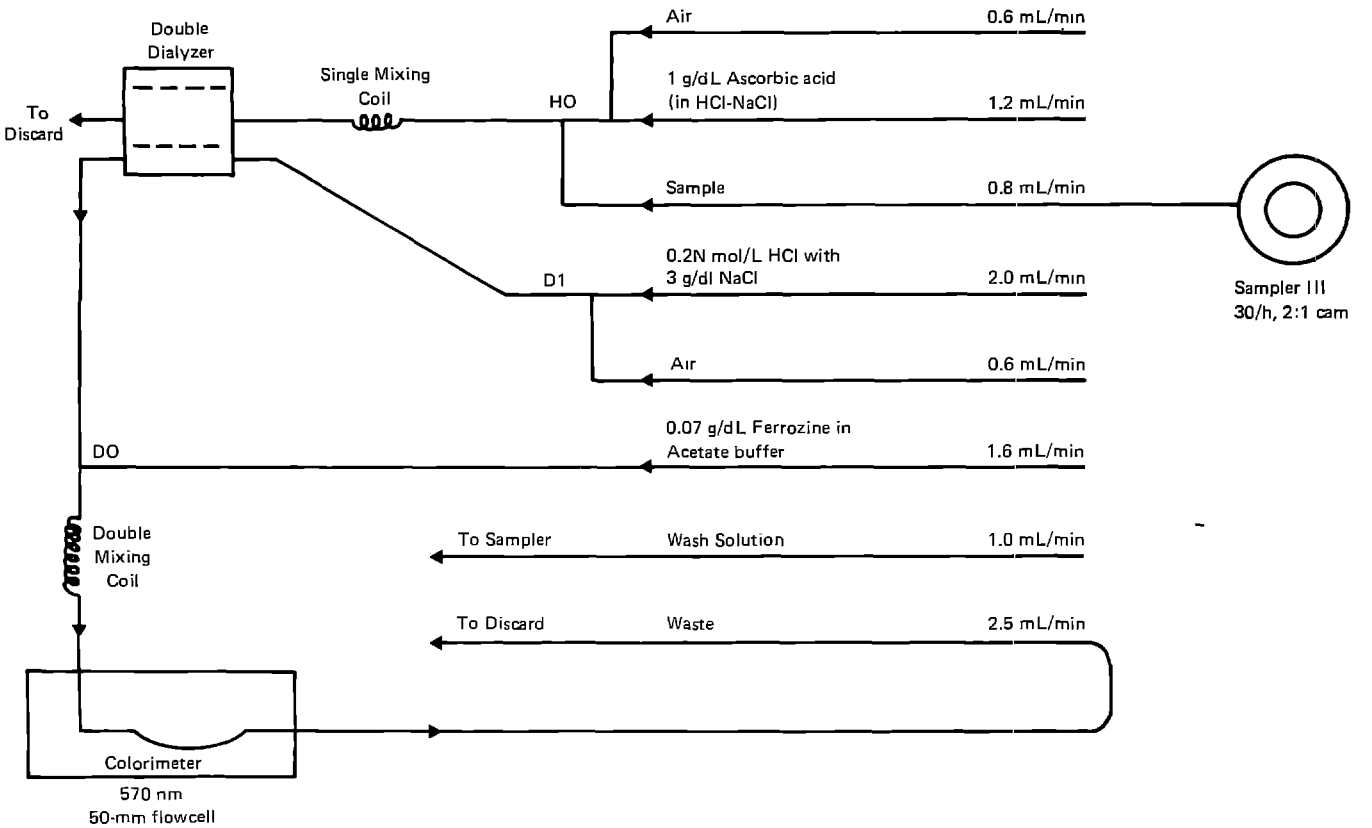
8. CDC Modifications

The following modifications to the Technicon AAII-25 method are noted: (a) The reagent concentrations used and their ratios are based on procedures developed at CDC, (b) two standard Technicon AutoAnalyzer I, type C, dialysis plate assemblies are connected in series to increase

the efficiency of dialysis, (c) ferrozine is incorporated into the acetate buffer, and (d) a 50-mm flowcell is used in the colorimeter to maximize sensitivity.

9. Flow Diagram

FIGURE 1. SERUM IRON AND TOTAL IRON-BINDING CAPACITY FLOW DIAGRAM



Section IV B References

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2. Ramsey WNM The determination of the total iron-binding capacity of serum. Clin Chem Acta 1957; 2:221.
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C. Serum Vitamins A and E

1. Principle

Vitamins A (retinol) and E (alpha-tocopherol) are measured in serum by an isocratic modification (*1*) of Bieri's high-performance liquid chromatography method (*2*). Serum is added to the internal standard solution, which contains retinyl acetate in ethanol. The ethanolic solution is extracted with hexane, and the extract is dried with nitrogen and redissolved in ethanol. An aliquot is injected onto a C_{18} reverse-phase radial-pack column and eluted with 95% methanol:5% water at 2.0 ml/min flow rate. Absorbance at 280 nm is recorded. Vitamins A and E are measured by comparing the height of the retinol or tocopherol peak to the height of the retinyl acetate internal standard peak. (Peak-area quantitation may also be used.)

2. Instrumentation

a. Waters HPLC system (Waters Associates, Milford, MA)
   (1) Model 6000A solvent delivery system
   (2) WISP 710B sampler
   (3) uBondapak C_{18} reverse-phase "rad-pak" column (10 cm)
   (4) Model 440 absorbance detector
   (5) Data module
   (6) System controller
   OR: Waters QA-1 HPLC with data module
b. Vortex mixer (Fisher Scientific Co., Fairlawn, NJ)
c. Sorvall GLC-1 centrifuge (Dupont-Sorvall Instruments, Newtown, CT)
d. Micromedic model 30010 high-speed pipettor (Micromedic Systems, Division of Rohm and Haas, Horsham, PA)
e. Cary 219 spectrophotometer (Varian Instruments, Palo Alto, CA) or: Acta CII and CIV double-beam spectrophotometers (Beckman Instruments, Fullerton, CA)
f. Micromedic APS-2 automatic pipetting station (Micromedic Systems)

3. Materials

a. N-Hexane, HPLC grade (Fisher Chemical Co., Fairlawn, NJ)
b. Ethanol, dehydrated, USP (U.S. Industrial Chemical Corp.)
c. Methanol, HPLC grade (Fisher Chemical Co.)
d. Nitrogen gas, high purity, 99.9% (Matheson Gas Co., East Rutherford, NJ)
e. Retinol (Sigma Chemical Co., St. Louis, MO)
f. Retinyl acetate (Sigma Chemical Co.)
g. d, L-Alpha tocopherol (ICN Biochemicals, Cleveland, OH)
h. L-Ascorbic acid, ACS grade (Fisher Chemical Co.)
i. 10- x 75-mm disposable glass culture tubes (Corning Glassworks, Corning, NY)
j. Pasteur pipettes, short tip (Corning Glassworks)
k. Sample vials and micro-inserts for the WISP (Waters Associates)

4. Reagent Preparation

a. *95% methanol:5% water mobile phase*
   Add 100 ml of deionized water to 1900 ml of HPLC-grade methanol and mix well. (One tray of 40 samples requires approximately 1300 ml of mobile phase at 2.0 ml/min.)
b. *10 g/dL ascorbic acid*
   Dissolve 10 g of l-ascorbic acid in deionized water in a 100-ml volumetric flask. Mix well and dilute to volume.

5. Standard Preparation

a. *Retinyl acetate internal standard*
   The concentration of the retinyl acetate internal standard is chosen to be 50-55 μg/dL, which is midrange peak height for normal vitamin A values. With the millimolar absorptivity of 1510 used for retinyl acetate, a 53 μg/dL solution has an absorbance of 0.08. A concentration stock solution is prepared each day of assay and diluted until its absorbance is 0.08.

   Dissolve 1-2 mg of all trans-retinyl acetate in ethanol, and dilute to volume in a 100-ml volumetric flask. Measure absorbance of this stock solution against a methanol blank at 325 nm. Divide the resulting absorbance by the desired absorbance 0.08 to get the appropriate dilution factor. For a working solution of 50-ml volume (enough for 250 samples), divide 50 ml by the dilution factor. Dilute this number of ml of stock with ethanol to 50 ml and recheck the absorbance. For example:

$$\frac{\text{Abs of stock solution } 1.432}{\text{Abs of final int. std. solution } 0.08} = 17.9 \text{ dilution factor}$$

$$\frac{50 \text{ ml volume int. std}}{17.9 \text{ dilution factor}} = 2.8 \text{ ml of stock needed}$$

After the absorbance of the internal standard is verified, add 0.5 ml (l%) of 10 g/dL ascorbic acid to prevent oxidation of the vitamins in the serum. (The additional dilution will be compensated for in the data module calculations.)

6. Procedure

a. Sample extraction

(1) Using the Micromedic APS-2 in sample transfer-single mode, pipette 200 μl of the serum specimen into 10- x 75-mm test tubes. Pipette 1 aliquot of each quality control pool for every 20 samples.

(2) Using the APS-2 reagent dispenser, add 200 μl of the internal standard solution containing retinyl acetate and ascorbic acid to each tube, and vortex tube for 10 sec.

(3) Using the high-speed dilutor, add 400 μl of hexane to each tube, and vortex for 45 sec to completely mix the layers.

(4) Centrifuge tubes for 1 min at 2000 RPM.

(5) Using a Pasteur pipette, carefully remove the hexane (upper) layer (approximately 300 μl) and transfer to a 10- x 75-mm glass tube.

(6) Place tubes in a 25°C water bath and evaporate hexane to dryness under a stream of nitrogen, using the specially designed manifold with ports for 20 samples.

(7) Remove tubes from bath and redissolve contents of each tube with 100 μl of ethanol. Mix each tube thoroughly, using the vortex mixer.

(8) Using a Pasteur pipette, transfer the contents of each tube into a prelabeled sample vial with a limited-volume glass insert, taking care to avoid introducing air bubbles at the bottom of the insert.

(9) Program the WISP, system controller, and data module for height measurements of the retinol, retinyl acetate, and a-tocopherol peaks. Begin sample injection.

7. Calculations

a. Quantitation of standards

Before making the standard curve, the three standards are purified by chromatographing each standard individually under the same conditions as the assay and collecting approximately the middle half of each peak. Starting optical density at the peak-absorbing wavelengths should be about 4.0, yielding an absorbance of about 0.4 after chromatography. In collecting the peak, a 2-sec lag time between residence in the detector cell and emergence must be taken into account. Criteria for purity are for

$$\text{vitamin A: } \frac{\text{Abs } 328}{\text{Abs } 315} = 2.1 \quad \frac{\text{Abs } 328}{\text{Abs } 280} = 4.6$$

$$\text{vitamin A acetate: } \frac{\text{Abs } 325}{\text{Abs } 315} = 2.1 \quad \frac{\text{Abs } 325}{\text{Abs } 280} = 4.5$$

$$\text{vitamin E: } \frac{\text{Abs } 292}{\text{Abs } 280} = 1.6$$

(Purified vitamin A and vitamin A acetate can be stored no more than 1 week at -70°C.) The absorbance of each standard is adjusted by dilution with ethanol to 0.240. At this absorbance, the concentrations will be:

$$\text{retinol—at 328 nm, } \frac{0.240}{1730\,\epsilon} = 134.8\ \mu\text{g/dL}$$

retinyl acetate—at 325 nm, $\frac{0.240A}{1510\,\epsilon} = 159\ \mu g/dL$

α-tocopherol—at 292 nm, $\frac{0.240A}{75.8\,\epsilon} = 3166.2\ \mu g/dL$

A solution is prepared with equal volumes of each standard. At this point, the standard concentrations are:

retinol—134.8 ÷ 3 = 44.9 μg/dL

retinyl acetate—159 ÷ 3 = 53 μg/dL

α-tocopherol—3166.2 ÷ 3 = 1055.4 μg/dL

Our daily working retinyl acetate internal standard concentration is 53 μg/dL; the calibration reference factor is therefore based on this concentration. Three 50-μL injections each are made on our Waters Tri-Module and QA-1 HPLC systems.
Retention times and reference factors are calculated and stored in memory in the calibration tables. Peak heights are measured either manually or by the 730 Data Module, and the factors (F_a, F_b) are calculated by the following equations:

$$\frac{\text{conc vit. A}}{\text{conc vit. Aac}} \times \frac{\text{pk ht vit. A}}{\text{pk ht vit. Aac}} = F_a$$

$$\frac{\text{conc vit. E}}{\text{conc vit. Aac}} \times \frac{\text{pk ht vit. E}}{\text{pk ht vit. Aac}} = F_b$$

b. Samples
Quantitation of the unknown samples is as follows:

$$\text{vit. A serum conc.} = \frac{\text{pk ht vit. A}}{\text{pk ht vit. Aac}} \times \text{conc. Aac} \times F_a$$

$$\text{vit. E serum conc.} = \frac{\text{pk ht vit. E}}{\text{pk ht vit. Aac}} \times \text{conc. Aac} \times F_b$$

Our experience has shown that a multilevel standard curve is always linear; therefore, we use only one point on the curve for determining F_a and F_b. If automated peak measurement and quantitation is chosen, the method is essentially the same as described above. The programming of the Waters equipment is outlined as follows: The three standards of known concentration are chromatographed as above. After chromatography, the 730 Data Module Calibration table is programmed as follows:

Parameter	Entry
45	0
10	1
11	peak no. (A = 1, Aac = 102, E = 3)
12	retention times of A, Aac, and E
13	concentrations of the three standards
14	response factors (to be calculated)
9	1
98	1 (response factors calculated)
9	0

Samples are then ready to run.
Other parameters are as usual, except peak width (parameter 21) should be reduced from its usual 13 to 5.

c. WISP programming
(1) Put on AUTO mode.
(2) Press SAMPLE NO button.

(3) Enter 0 as sample no.
(4) Press ENTER button.
(5) Press INJ VOL button.
(6) Enter 50 as injection volume (μL).
(7) Press ENTER button.
(8) Press RUN TIME button.
(9) Enter 20 as run time (min).
(10)Press ENTER button.
(11)Press ENTER button twice.

d. System controller programming
(1) System Monitor
(a) Page 1 Date xxxxxx time hr-min-sec operator xx
(b) Page 2 op. no.: 01 Time: (leave blank)
Vials from: 01 through: 48 (for 1 full tray)
Method: 01
(c) Page 3 Method No.: 01 Method Name: (leave blank)
Pump Set: 01 Column: C18
Detector: 280
(*NOTE*: Rest of pages of system monitor need not be programmed.)

(2) Pump monitor
(a) Page 1 No programming
(b) Page 2 Flow:2 %A:100 %B:0 %C:0
(c) Page 3

Time	Flow	%A	%B	%C	Curve
Initial	2	100	0	0	*
25	0	100	0	0	11

(d) Pump control: No programming. Leave as preset.

e. Data module programming (except internal standard programming)

Parameter No.	Parameter	Parameter Value
0	Date	03-12-82
1	Time	10:03:00
2	Chart speed	0.4 cm/min
3	Plot mode	0
4	Pen 2 on/off	0
5	Pen 1 zero	10
6	Pen 2 zero	0
7	Auto zero	1
9	Calibration analysis	0
10-14	See internal standard programming	
20	Auto parameter	0
21	Peak width	5
22	Noise rejection	30
33	Report format	10,000
34	Sample no. to start with	0
60	Peak height	1
65	Peak height at internal standard (from calibration)	
72	Double-peak width	7.0
74	Noise rejection double	7.01
81	Inhibit integration	0.01
82	Resume integration	2.50

Section IV C References

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D. Plasma Glucose

1. Principle

Glucose is measured by a micro adaptation of the National Glucose Reference Method (*1*) on a Gilford System 3500 Computer-Directed Analyzer (*2*). The determination is based on the enzymatic coupling of hexokinase and glucose-6-phosphate dehydrogenase (G-6-PD), and it has been optimized for D-glucose. Specificity is enhanced by using sample deproteinization with barium hydroxide and zinc sulfate (Somogyi reagents) to remove kinases and oxidoreductases that use coenzymes, carbohydrate modifying enzymes, ultraviolet absorbing proteins, and other possibly interfering chemicals that coprecipitate.

Hexokinase and excess adenosine triphosphate (ATP) are added to the supernatant, and—in the presence of magnesium ions—they phosphorylate the glucose to glucose-6-phosphate (G-6-P). In the presence of nicotinamide adenine dinucleotide (NAD), G-6-P is oxidized by G-6-PD to 6-phosphoglucono-Δ-lactone and nicotinamide adenine dinucleotide hydrogenase (NADH). Spontaneous hydrolysis of the unstable lactone occurs at the pH of the test, and the reaction sequence goes virtually to completion. The glucose in the filtrate is measured by the reduction of NAD to NADH measured at 340 nm.

$$\text{D-glucose} + (\text{ATP Mg}) = \text{D-glucose} + (\text{ATP}\cdot\text{Mg}) \xrightarrow{\text{hexokinase}} \text{D-glucose-6-phosphate} + (\text{ADP}\cdot\text{Mg})^- + \text{H}^+.$$

$$\text{D-glucose-6-phosphate} + \text{NAD} + \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}} \text{6-phosphoglucono - }\Delta\text{-lactone} + \text{NADH} + \text{H}^+.$$

$$\text{6-phosphoglucono-}\Delta\text{-lactone} + \text{H}_2\text{O} \longrightarrow \text{6-phosphogluconic acid.}$$

2. Instrumentation

a. Gilford System 3500 computer-directed analyzer (Gilford Instrument Laboratories, Oberlin, OH)
b. Micromedic model 25000 automatic pipettes (2) with 200-μL sampling and 5.0-mL dispensing pumps (Micromedic Systems, Division of Rohm and Haas, Horsham, PA)
c. Sorvall GLC-1 centrifuge (Dupont-Sorvall Instruments, Newtown, CT)
d. Beckman research model 1019 pH meter (Beckman Instrument Co., Fullerton, CA)
e. Mettler model H-16 analytical balance (Mettler Instrument Co., Princeton, NJ)
f. Acta-CV double-beam spectrophotometer (Beckman Instruments Co.)
g. Vortex mixer (Fisher Scientific Co., Fairlawn, NJ)

3. Materials

a. D-glucose, SRM No. 917 (National Bureau of Standards, Washington, DC)
b. Benzoic acid, primary standard, meets ACS specifications (J.T. Baker Co., Phillipsburg, NJ)
c. Zinc sulfate, heptahydrate, meets ACS specifications (Merck and Co., Rahway, NJ)
d. Barium hydroxide, octahydrate, meets ACS specifications (J.T. Baker Co.)
e. Magnesium acetate, tetrahydrate, meets ACS specifications (J.T. Baker Co.)
f. Tris (hydroxymethyl) aminomethane (Tris base), reagent grade, 99.9% purity (Sigma Chemical Co., St. Louis, MO)
g. Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), reagent grade (Sigma Chemical Co.)
h. Nicotinamide adenine dinucleotide, oxidized (NAD^+), dihydrate, grade V: 99+% by formula weight (Sigma Chemical Corp.)
i. Adenosine 5'-triphosphate (ATP) disodium salt, trihydrate, purity of 98% or higher on the basis of formula weight (ICN Pharmaceuticals, Cleveland, OH)
j. Hexokinase (HK), from yeast, highly purified (Boehringer-Mannheim Co., Indianapolis, IN)
k. Glucose-6-phosphate dehydrogenase (G-6-PD) from *Leuconostoc mesenteroides*, highly purified (Boehringer-Mannheim Co.)
l. Bovine serum albumin, Pentex, fraction V, 96-99% pure (Miles Laboratories, Elkhart, IN)
m. Glucose-l-phosphate, disodium salt, tetrahydrate, 98% pure (Boehringer-Mannheim Co.)
n. D-fructose, meets NAS/NRC specifications (J.T. Baker Co.)

4. Reagent Preparation

a. *2.5 g/L Benzoic acid*
Dissolve—by heating—1.0 g of benzoic acid in 500 mL of distilled water in a 1-L volumetric flask. After dissolution is completed, dilute to volume with distilled water.

b. *22 g/L Zinc sulfate solution*
Dissolve 22.0 g of zinc sulfate ($ZnSO4.7H_2O$) in 900 mL of hot, CO_2-free water. Cover the container with a watch glass while the solution cools, transfer to a 1-L volumetric flask, dilute to volume with CO_2-free water, and mix well. Store in a tightly stoppered, glass reagent bottle.
NOTE: Reagents used for sample deproteinization, including the zinc sulfate and both barium hydroxide solutions, are prepared with CO_2-free water (distilled water that has been vigorously boiled to expel dissolved CO_2).

c. *Saturated barium hydroxide*
Using a freshly opened bottle of barium hydroxide ($Ba(OH)_2.8H_2O$), dissolve 80 g in approximately 900 mL of hot, CO_2-free water. Cover the container with a watch glass while the solution cools, transfer the solution to a 1-L volumetric flask, dilute to volume with CO_2-free water, and mix well.

d. *0.055 mol/L Barium hydroxide*
Without disturbing the precipitate, transfer 245 mL of saturated barium hydroxide to a 1-L volumetric flask. Dilute to volume with CO_2-free water. Titrate 10.0 mL of zinc sulfate solution with this diluted barium hydroxide solution to a faint pink end point with phenolphthalein indicator (2 drops 0.5 g/dL indicator in 95% ethanol). Ideally, 10.0 mL of zinc sulfate should require 10.0 ± 0.1 mL of barium hydroxide solution.
NOTE: If this limit is exceeded, add either saturated barium hydroxide or CO_2-free water in the appropriate calculated quantities to the 0.055 mol/L $Ba(OH)_2$ and repeat the titration. Transfer the adjusted $Ba(OH)_2$ solution to a glass reagent bottle fitted with a soda-lime trap and a syphon or decanting tube system. Each month check equivalence of this solution by titration.

e. *Tris-HCl stock solution*
Dissolve 31.52 g of Tris-HCl in distilled water in a 2-L flask. Mix well and dilute to volume.

f. *Tris base stock solution*
Dissolve 6.06 g of Tris base in distilled water in a 500-mL volumetric flask. Mix well and dilute to volume.

g. *0.1 mol/L Tris-magnesium buffer, pH 7.5 at 25° C*
Mix 800 mL of Tris-HCl solution and 200 mL of Tris base solution; dissolve 1.1 g of magnesium acetate in the solution. Determine pH of the solution of 25°C. If necessary, adjust pH with either Tris-HCl or Tris base to pH 7.5 ± 0.1. Filter the solution through a sterile 0.45 μ-membrane filter into a sterilized borosilicate-glass, screw-cap storage bottle. (Stable for up to 6 mo at 4°C if carefully handled.)

h. *Tris-albumin*
Dissolve 0.5 g of bovine serum albumin in Tris-magnesium buffer in a 250-mL volumetric flask. Mix well and dilute to volume. (Store at 4°C.)

i. *Stock hexokinase solution*
Weigh or measure volumetrically an amount of hexokinase estimated to have a total activity of about 1250 IU at 25°C. Transfer this amount to a 250-mL volumetric flask and dilute to volume with Tris-magnesium buffer. (Store at 4°C and assay on day of preparation as described on pages 102 and 103 of reference 1.)

j. *Stock glucose-6-phosphate dehydrogenase solution*
Weigh or measure an amount of G-6-PD estimated to have a total activity of 1250 IU at 25°C. Transfer this to a 250-mL volumetric flask and dilute to volume with Tris-magnesium buffer. (Store at 4°C and assay on day of preparation as described on pages 103 and 104 of reference 1.)

k. *Stock NAD solution*
Dissolve 0.9952 g of nicotinamide adenine dinucleotide in Tris-magnesium buffer in a 250-mL volumetric flask. Dilute to volume. (Store at 4°C and assay on day of preparation as described on pages 104 and 105 of reference 1.)

l. *Stock ATP solution*

Dissolve 0.826 g of adenosine triphosphate, disodium salt, in Tris-magnesium buffer in a 250-mL volumetric flask. Dilute to volume. (Store at 4°C and assay on day of preparation as described on page 105 of reference 1.)

m. *Working enzyme reagent*

After all enzyme and coenzyme activities have been determined, add to a 1-L volumetric flask the following amounts of these four components:

(1) Hexokinase—amount of stock solution equal to 800 IU
(2) G-6-PD—amount of stock solution equal to 800 IU
(3) NAD—200 ml of stock with concentration greater than or equal to 0.0045 mM/mL
(4) ATP—200 mL of stock with concentration greater than or equal to 0.0045 mM/mL

Dilute to 1.0 L with Tris-magnesium buffer and mix contents thoroughly by inversion. Immediately after enzyme reagent is prepared, dispense 100 mL into each of 10 sterile, dry, screw-cap 125-mL heavy borosilicate glass bottles; store at -20°C until used. (Stable for 6 months.) On the day of assay, remove the enzyme reagent from the freezer and place in a 25°C water bath or allow to thaw at room temperature. Before using a new enzyme reagent, test a portion of it for adequacy by following the procedures described on pages 106 and 107 of reference 1. Reagent that does not meet the criteria for adequacy cannot be used in this procedure.

NOTE: The actual composition of the working enzyme reagent is based upon the assays of the four stock solutions above (i through l).

5. Standard Preparation

a. *300 mmol/L (5400 mg/dL) Stock glucose standard*

Dissolve 5.400 g of NBS D-glucose (dextrose) in 2.5 g/L benzoic acid in a 100-mL volumetric flask. Mix well and dilute to volume. Store at -20°C in 20-mL aliquots in tightly capped containers for preparing working standards. (Prepare a new stock every 6 mo.)

b. *Working glucose standards*

Using a 30-mL aliquot of thawed and well-mixed stock standard, prepare the following dilutions, bringing each to 100-mL volume with 2.5 g/L benzoic acid:

mL Glucose Stock Standard	mmol/L Concentration	mg/dL Concentration
3.0	9	162
5.0	15	270

NOTE: Store working standards in tightly capped bottles at 4°C. For use, mix and pour an aliquot for sampling; never insert pipettes into the standard containers. If specimens with elevated glucose levels are to be analyzed, higher concentrations of standards should be included; the method is linear to 600 mg/dL (33.3 mmol/L). A 600 mg/dl standard, prepared by diluting 11.1 mL of stock glucose standard to 100 mL with benzoic acid diluent in a volumetric flask, is routinely analyzed at the beginning of each analytical day as a part of the enzyme reagent and instrument linearity check.

6. Procedure

a. *Filtrate preparation*

(1) Bring standards, quality control materials, and plasma specimens to room temperature. Mix each well.
(2) Using a Micromedic automatic pipette, dilute 0.2 mL of each sample with 2.0 mL of 0.055 mol/L barium hydroxide into a 16- X 125-mm disposable screw-cap tube.
(3) Immediately dispense 2.0 mL of zinc sulfate solution into the same tube, using a second Micromedic pipette. Cap tube and mix contents using a vortex mixer for 5 sec.
(4) After all filtrates have been prepared, remix each tube. Centrifuge all tubes at 2000 RPM for 20 min.

(5) Decant supernates into labeled smaller tubes and recentrifuge. Samples are now ready for analysis.

b. *Calculations*

Gilford System 3500 parameters, analytical procedure, calculations, and maintenance are as described in the hexokinase procedure of the Instruction Manual, with the following exception: the Gilford System 3500 procedure is based on direct analysis of serum or plasma; in this method the calibration factor is determined by using aqueous glucose standards taken through the entire procedure.

NOTE: The enzymatic reaction can also be performed manually by adding one part of supernatant to five parts of enzyme reagent, mixing, allowing 10 to 20 min for the reaction to go to completion, then recording absorbance on a good-quality spectrophotometer at 340 nm versus distilled water. Calculations are performed by using the linear regression of the slope and intercept of the standard curve.

Section IV D References

1. Neese JW, Duncan P, Bayse D, Robinson M, Cooper T, Stewart C. Development and evaluation of a hexokinase/glucose-6-phosphate dehydrogenase procedure for use as a national glucose reference method. Atlanta: Center for Disease Control, GA, 1976. DHEW publication no. (CDC) 77-8330.
2. Gilford Laboratories, Inc. Instruction manual for the Gilford system 3500 computer-directed analyzer. Oberlin, Ohio: Gilford Laboratories, Inc, June 1978.

E. Serum and Red Cell Folate

1. Principle

Serum and red cell folic acid are measured by using the Bio-Rad Laboratories' "Quanta-Count Folate" radioassay kit (*1*), which is based on assays described by Dunn and Foster (*2*) and Waxman and Schreiber (*3*). Serum (or whole blood diluted 1:5 with 1.0 g/dL ascorbic acid) is mixed with a pH 9.4 borate-dithiothreitol buffer and radioactively labeled folate (^{125}I-pteroylglutamic acid) derivative. After the serum is heated, folate-binding protein is inactivated while the folate is stabilized by the buffer. A binding material, folate-binding milk protein (lactalbumin), is added to the mixture of ^{125}I-labeled and -unlabeled (serum) folate in a quantity sufficient to bind some, but not all, of the folate present. During incubation, the labeled and unlabeled folates compete for the binding sites available on the folate-binding protein on the basis of their concentrations. The higher the folate concentration in the serum, the more unlabeled folate it contains, and therefore less ^{125}I-labeled folate will bind to the binding protein. The bound and free (unbound) folate is separated after incubation by using dextran-coated charcoal. The level of ^{125}I-labeled folate is measured by using an LKB Rackgamma II gamma counter. The higher the level of radioactivity, the more ^{125}I-folate has been bound and the less unlabeled folate originally present in the serum (or whole blood).

2. Instrumentation

a. LKB Rackgamma II Gamma Spectrometer (LKB Instruments Co., Gaithersburg, MD)
b. IEC Centra-7 centrifuge (International Equipment Co., Needham Heights, MA)
c. Vortex mixer (Fisher Scientific Co., Fairlawn, NJ)
d. Thelco model 84 water bath (Precision Scientific Co., Chicago, IL)
e. Eppendorf pipettors, 50-1000 μL sizes (Brinkmann Instruments, New York, NY)

3. Materials

a. Bio-Rad Laboratories Quanta-Count Folate radioassay kit, 200-test size (Bio-Rad Laboratories, Richmond, CA)
b. Red cell folate reagent pack (lyophilized folate-free protein base and preservative) (Bio-Rad Laboratories)
c. L-ascorbic acid, 99% purity (J.T. Baker, Phillipsburg, NJ)
d. Disposable 12- x 75-mm polypropylene tubes, #T1226-12 (American Scientific Products, McGaw Park, IL)

4. Reagent Preparation

a. Reconstitute the following reagents as directed in the Quanta-Count Folate Instruction Manual:

(1) *Folate binding protein stock solution*—Add 10.0 mL of distilled water. (Store at -20°C if not completely used on day of analysis.)

(2) *Dithiothreitol (DTT)*—Add 4 mL of borate buffer.

(3) *Folate (PGA)^{125}I derivative stock trace solution*—Add 1.3 mL of distilled water. (Store at 2-8°C. Less than 10 μCi activity as ^{125}I-PGA.)

(4) *Red cell folate diluent*—Add 5.0 mL of distilled water. (Store at -20°C if not completely used on day of analysis.)

b. Using these stock solutions, prepare the following reagents:

(1) *Folate binding protein working solution*
Dilute 1 part of stock solution with 4 parts of distilled water; 1.0 mL of working solution will be required for each specimen or standard assayed.

(2) *Dithiothreitol-borate buffer, pH 9.4*
Add the remainder of the borate buffer to the DTT and mix. (Store at 2.8°C.)

(3) *Working tracer solution*
Dilute the ^{125}I-PGA stock tracer solution 1:100 with DTT-buffer; that is, for every tube analyzed, dilute 10 μL of tracer to 1 mL with buffer. (Each standard, control, and specimen requires two tubes; two blank tubes and one total counts tube are also required.)

5. Standard Preparation

Reconstitute each of the serum-based PGA standards (1.0, 2.5, 5.0, 10.0, and 20.0 ng/mL) with 1.0 mL of distilled water. Reconstitute the "P-Zero" standard with 2.0 mL water. (If all of the kit is not to be used in one day, store aliquots of the standards at -20°C.)

6. Procedure

a. Sample preparation
Perform the analysis for serum as described in the Quanta-Count Folate Instruction Manual. For whole blood analysis, upon collection, dilute 500 μL of whole blood with 2.0 mL of 1.0 g/dL ascorbic acid and freeze specimen. Before assaying, thaw the hemolysate, mix well, and dilute 500 μL of hemolysate with 600 μL of 1.0 g/dL ascorbic acid to give a 1:11 dilution of the whole blood. Then dilute 200 μL of this solution with 200 μL of the red cell protein diluent to give a final dilution of 1:22. Use 100 μL of the final solution to perform the assay.

NOTE: In HANES, the 1:5 hemolysate dilution is prepared in the field, and the specimen is immediately frozen and shipped on dry ice.

b. Analysis

(1) Label two 12- X 75-mm reaction tubes for the blank, two for each standard, including the P-Zero, and two for each serum or red cell specimen.

(2) To the blank tubes, add 100 μL P-Zero standard. To the standard tubes, add 100 μL of the appropriate standard—P-Zero, P-1.0, P-2.5, P-5.0, P-10.0, and P-20.0. Add 100 μL of each participant's sample to the appropriate tubes.

(3) To all tubes, add 1.0 mL of working tracer solution (prepared immediately before the assay). Mix all tubes gently.

(4) Prepare a total counts tube by adding 1.0 mL of working tracer solution. Set aside until tubes are to be placed in the counter.

(5) Place the tube rack containing all of the tubes in a boiling water bath for 17.5 min. (This allows the bath to return to 100°C for 15 min after cover is replaced on bath.) Cap tubes loosely. Cool to room temperature by placing rack in a room temperature bath.

(6) Add 1.0 mL of distilled water to the blank. Add 1.0 mL of folate-binding protein working solution to all other tubes. Mix all tubes well.

(7) Incubate tubes in a room temperature bath for 30 min.

(8) After incubation, add one charcoal adsorbent tablet to each tube and allow tube to stand for 5 min. Vortex each tube for 10 sec, and allow to stand for 5 min.

(9) Centrifuge all tubes for 10 min at 2000-3000 rpm to pack the adsorbent.
(10) Decant the supernatants into appropriately labeled counting vials.
(11) Count each supernatant and the total counts tube in the LKB Rackgamma II for 1 min.

7. Calculations

The LKB Rackgamma II has full data-reduction capabilities. Method 4 (logit B/B_0 vs. $\log_{10}$ concentration) is used, where logit $(B/B_0)=Ln((B/B_0) / (1-B/B_0))$, B = corrected counts/min for each tube, and B_0 = nonspecific binding in the 0 standard. This method results in a linearized standard curve with an inverse relationship of levels of radioactivity to concentration of folate (as pteroylglutamic acid). Serum results are expressed as nanograms folate per milliliter of serum (ng/mL). Red cell folate values are multiplied by 22, the dilution factor of the whole blood. The serum folate level multiplied by (1.0-hematocrit) is subtracted, and the resulting value is divided by the hematocrit to give ng/mL red cell folate:

$$\text{RBC folate} = \frac{\text{whole blood folate - serum folate (1-hematocrit/100)}}{\text{hematocrit X 100}} = \text{ng/mL RBC}$$

8. CDC Modifications

The following modifications are noted to the Bio-Rad Instruction Manual: (a) because the HANES specimens are collected in the field and shipped frozen, the 1:22 whole blood dilution is prepared from a 1:5 hemolysate rather than directly, (b) 1.0 g/dL ascorbic acid rather than 0.4 g/dL is used as the diluent, and (c) whole blood with diluent is not incubated, since a freeze-thaw cycle accomplishes maximum red cell conjugase-activating effect, as described by Mortensen (*4*) and Netteland and Bakke (*5*).

Section IV E References

1. Bio-Rad Laboratories. Instruction manual· Bulletin 4201: Quanta-Count Folate. Richmond, California: Bio-Rad Laboratories, October 1981.
2. Dunn RT, Foster LB: Radioassay of serum folate. Clin Chem 1973; 19 1101-5.
3. Waxman S, Schreiber C. Measurement of serum folate levels and serum folic acid binding protein by ^{3}H-PGA radioassay. Blood 1973; 42:281-93.
4. Mortensen E. Effect of storage on the apparent concentration of folate in erythrocytes as measured by competitive protein binding radioassay. Clin Chem 1978; 24:663-8.
5. Netteland B, Bakke OM. Inadequate sample-preparation technique as a source of error in determination of erythrocyte folate by competitive binding radioassay. Clin Chem 1977; 23:1505-6.

F. Whole Blood Lead

1. Principle

Lead is measured in whole blood by atomic absorption spectroscopy by using a modification (*1*) of the Delves method (*2*). Quantitation is based on the measurement of light absorbed at 283.3 nm by ground state atoms of lead from a lead hollow-cathode lamp source. Whole blood samples, bovine whole blood quality controls, and standards (bovine whole blood spiked with aqueous lead standards) are diluted with nitric acid as the oxidizing agent, dried, and ashed, and lead content is determined by using a Perkin-Elmer Model 360 or Model 2380 atomic absorption spectrophotometer with deuterium background correction. All materials used for collecting and processing specimens are screened for possible lead contamination, and all processing work, except ashing, is performed under laminar-flow hoods.

2. Instrumentation

a. Perkin-Elmer models 360 and 2380 atomic absorption spectrophotometers with deuterium arc background correction, lead hollow-cathode lamp, and a micro-combustion assembly (Perkin-Elmer Corp., Norwalk, CT)

Parameter	Setting
Wavelength	283.3 nm
Lamp current	10 mA
Slit	0.7 nm (normal mode)
Signal	TC-1 (time constant-1)
Operating mode	CONC (concentration)

b. Perkin-Elmer model 56 recorder; range: 10 mV; chart speed: 10 mm/min (Perkin-Elmer Corp.)
c. Sample tray holder (20-cup capacity) and nickel Delves micro-cups (Perkin-Elmer Corp.)
d. Gravity-type oven, set at 130-140°C (Blue M Electric Co., Blue Island, IL)
e. Corning model PC-35 ceramic-top heating plate, setting at 5.4 = approx. 370°C (Corning Glassworks, Corning, NY)
f. Heater control plate (Perkin-Elmer Corp.)
g. Micromedic "Digiflex" automatic pipette, with 20-μL sampling and 200-μL dispensing pumps (Micromedic Systems, Div. of Rohm and Haas, Horsham, PA)
h. Model D-003 ceramic high-temperature absorption tubes (Trace Metals Instruments, Inc., New York, NY)
i. Cooling cabinet for samples (stainless steel and glass) (Brinkmann Instruments, Westbury, NY)
j. EACI model 100-plus modular unit vertical laminar-flow hood (Environmental Air Control, Inc., Hagerstown, MD)
k. Vortex mixer (Fisher-Scientific Co., Fairlawn, NJ)

3. Materials

a. Lead nitrate, SRM No. 928 (National Bureau of Standards, Washington, DC)
b. Redistilled concentrated nitric acid (G. Frederick Smith Chemical Co., Columbus, OH)
c. Acetylene, 99.6% purity (Matheson Gas Co., East Rutherford, NJ)
d. Bovine whole blood (EDTA as anticoagulant), from undosed cows for standard additions (5-10 μg/dL), and from lead-dosed cows (20-65 μg/dL) for various levels of quality control pools

4. Reagent Preparation

a. *0.5% (v/v) Nitric acid*
Dilute 5 mL of concentrated, redistilled nitric acid to volume in a 1-L volumetric flask with deionized water and mix well. (Prepare as needed.)
b. *1.0% (v/v) Nitric acid*
Dilute 10 mL of concentrated, redistilled nitric acid to volume in a 1-L volumetric flask with deionized water and mix well. (Prepare as needed.)

5. Standard Preparations

a. *1000 mg/mL Stock lead standard*
Transfer 1.5985 g of NBS lead nitrate to a 1-L volumetric flask. Dissolve lead nitrate with 1% nitric acid; dilute to volume with additional 1% nitric acid. (Prepare every 6 mo; store in polyethylene container.)
b. *10 mg/mL Intermediate lead standard*
Transfer 1.0 mL of stock standard to a 100-mL volumetric flask and dilute to volume with 0.5% nitric acid. (Prepare on day of analysis.)
c. *Working lead standards*
Transfer the following volumes of intermediate standard to 100-mL volumetric flasks and dilute to volume with 0.5% nitric acid:

Intermediate Stock (mL)	Working Standard Concentration (μg/dL)
0	0
1.0	10
2.0	20
4.0	40
6.0	60
8.0	80

(Prepare on day of analysis. See 6.b. for method of spiking blood with aqueous lead standards.)

6. Procedure

a. Sample preparation

(1) Allow frozen whole blood specimens and quality control blood specimens to reach ambient temperature and mix well (vortex) at least 30 sec, especially if sample has been frozen for some time. Before sampling, invert each tube five times. Prepare 40 specimens in duplicate for 1 day's analysis.

(2) With the Micromedic Digiflex pipette in the "sample transfer" mode, aspirate 10 μL of blood into delivery tip. Dispense sample into a sample cup held in the sample tray. In the "dispense" mode, dispense 150 μL of nitric acid into the sample cup.

(3) As each 20-sample tray is filled, place it in the drying oven at 130-140°C and dry blood samples for 15-30 min. After the drying procedure, place tray on the heater control plate (on the hot plate) and ash samples at about 370°C for at least 15 min. Be sure sample cups rest flat against heating block. The samples must be completely ashed to prevent falsely elevated results; this is critical to the assay. After ashing, remove tray and allow it to cool in the cooling cabinet.

b. Spiked-blood standard preparation

(1) With the Micromedic Digiflex pipette, aspirate 10 μL of EDTA-anticoagulated bovine whole blood (from nondosed cows) and dispense with 50 μL of 0.5% nitric acid into sample cups.

(2) Aspirate 10 μL of the 0 μg/dL lead standard and dispense with 100 μL of 0.5% nitric acid into a sample cup. Repeat procedure with remaining standards in their respective cups, preparing five sets of standards (0-80 μg/dL) for 1 day's analysis, to bracket four sets of 20 samples.

(3) Dry and ash the standard-blood mixtures in the same manner as samples, and allow cups to cool.

c. Instrument preliminary calibration for the Model 360

(1) Set instrument parameters as in 2.a. Turn instrument and lamp on.

(2) Set instrument in "energy" mode, and using "gain" knob, set "energy" meter readout to 50.

(3) Peak wavelength with "fine adjust" knob to maximize "energy" reading.

(4) Reset "energy" to 50 if necessary.
(For the Model 2380, use the "lamp" setting and set lamp current at 10 mA. In "set-up" mode, peak energy using the wavelength fine adjustment knob. Then turn to "abs" mode and turn on deuterium background correction with the "AA-EN" setting. All other instructions will be identical to the 360.)

(5) With flame off, check alignment of absorption tube, (still in "energy" mode) for maximum signal.

(6) Reset instrument to "absorbance" mode and depress autozero button to zero meter.

(7) Align absorption tube and holder with "in," "out," "up," and "down" adjustment knobs to maintain zero reading on meter. Check placement of cup holder under aperture of absorption tube.

(8) Turn on gas from tank. (Gas pressure to instrument should be 10 psi.) Make sure the drainage loop from the burner contains water.

(9) Turn on air. (Compressed air, whether tank or house-supplied, should be at 40 psi.) Press "ignite" button and wait for it to light. Lift fuel toggle switch and light burner head. (If lighting of flame is unsuccessful, check gas gauge to make sure acetylene pressure is 10 psi.)

(10) Allow flame to burn and instrument to warm up for 10-20 min, with the nebulizer aspirating deionized water. Make sure water is running freely through drainage tube.

(11) Adjust the flow rates of acetylene and air slightly to give a flame that appears almost completely blue with only slight vertical streaks of orange.

(12) Make the final alignment check of the sample cup in place under the aperture of the absorption tube by igniting cups containing the 60 μg/dL lead standard (dried and ashed in the base-blood matrix). In the "absorbance" mode, after all alignments are properly made, these standards should read at least 0.15-0.20 absorbance units to proceed. If not, recheck all alignment steps.

d. Operation

(1) If absorbance readings for 60 μg/dL standards are satisfactory, place instrument in "concentration" mode, and begin flaming sample cups.

(2) Place cooled sample cup into cup-loop of the microcombustion assembly and introduce the cup into the flame.

(3) Proceed with the sample sequence, alternating sets of standards with trays of 20 samples (bracketing) until all cups have been analyzed.

(4) Record absorbance readings corresponding to peak heights on the recorder.

7. Calculations

a. Correct the absorbance values of the blood standards by subtracting the absorbance of the unspiked blood standard from the absorbances of the lead-spiked blood standards.

b. Calculate the concentration of each specimen from the slope and y-intercept of the averaged standard curve values that bracketed each tray of samples, using this format:

(1) Standard set I
(2) Sample set A, replicate 1
(3) Standard set II
(4) Sample set B, replicate 1
(5) Standard set III
(6) Sample set A, replicate 2
(7) Standard set IV
(8) Sample set B, replicate 2
(9) Standard set V

Standard sets I and II are averaged and used in calculating sample set A-1; sets II and III are averaged and used in calculating sample set B-1, and so on. The replicate sample results are averaged to give a final concentration value.

All four runs are calculated by using a linear regression computer program, which generates slopes, intercepts, correlation coefficients, standardized residuals, and plotted and fitted curves. Base pool levels are calculated for each run. Individual curve points more than ± 2.58 standard deviations away from the best-fitted line can be validly deleted and the linear regression recalculated. "R^2" or the correlation coefficient squared for each run should be .94 or more. Slopes should be more than 0.0045; intercepts should be less than 0.01.

c. Repeat specimen analysis when duplicate absorbances or concentration values differ by more than 0.025A or 7 μg/dL. Reanalyze specimens containing more than 30 μg/dL lead for confirmation. For those specimens that have values greater than 80 μg/dL, use a 5-μL sample size.

8. CDC Modifications

The following modifications to the original method are noted: (a) nitric acid is used rather than hydrogen peroxide as the oxidizing agent, (b) drying and ashing constitute two steps rather than one, (c) a deuterium background corrector is used to compensate for refractory blood

components such as sodium chloride, which can give a small, nonspecific absorption signal when blood is being analyzed at the 283 nm lead wavelength; and (d) experientially, we have found that the Delves sample cups should be reused no more than 10 times to minimize imprecision errors, and that alignment of all parts of the Delves assembly is critical for maximum sensitivity in analysis.

Section IV F References

1. Barthel WF, Smrek AL, Angel CP, et al. Modified Delves cup atomic absorption determination of lead in blood. J Assoc Off Anal Chem 1973; 56:1253-6.
2. Delves HT. A micro-sampling method for the rapid determination of lead in blood by atomic-absorption spectroscopy. Analyst 1970; 95:431-8.

G. Serum Ferritin

1. Principle

Serum ferritin is measured by using the Bio-Rad Laboratories "Quantimune Ferritin IRMA" kit (*1*), which is a single-incubation two-site immunoradiometric assay (IRMA) based on the general principles of assays as described by Addison et al. (*2*) and Miles (*3*) and modified by Jeong et al. (Jeong H, Blackmore J, Lewin N. U.S. Patent No. 4,244,940). In this IRMA, which measures the mostly basic isoferritin found in serum, highly purified ^{125}I-labeled antibody to ferritin is the tracer, and the ferritin antibodies are immobilized on polyacrylamide beads as the solid phase. Serum or ferritin standards (made from human liver) are mixed with the combined tracer/solid-phase antibody reagent, and the mixture is incubated. During incubation, both the immobilized and the ^{125}I-labeled antibodies bind to the ferritin antigen in the serum or standards, thus creating a "sandwich."

After incubation, the beads are diluted with saline, centrifuged, and decanted. The level of ^{125}I-labeled ferritin in the pellets is measured by using a gamma counter. There is a direct (rather than inverse, as in most RIA) relationship between the radioactive levels of the pellets and the amount of endogenous ferritin in the serum or standards.

2. Instrumentation

a. LKB Rackgamma II gamma spectrometer (LKB Instruments Co., Gaithersburg, MD)
   or:
   Micromedic Apex Automatic Gamma Counter (Micromedic Division, Rohm and Haas, Horsham, PA)
b. Sorvall RC-2B refrigerated centrifuge (Dupont Sorvall Instruments Co., Newtown, CT)
c. Vortex mixer (Fisher Scientific Co., Fairlawn, NJ)
d. Thelco model 84 water bath (Precision Scientific Co., Chicago, IL)
e. Micromedic autodiluter, with 100 μL and 500 μL pumps (Micromedic Systems, Div. of Rohm and Haas, Horsham, PA)
f. Eppendorf pipettors, 500-200 μL sizes (Brinkmann Instruments, New York, NY)

3. Materials

a. Bio-Rad Laboratories "Quantimune Ferritin IRMA" ferritin assay kit, 200-test size (Bio-Rad Laboratories, Richmond, CA)
b. "Lyphochek" 3-level ferritin quality control materials and "Lyphochek Anemia Control" material (ECS Division of Bio-Rad Laboratories)
c. Disposable 12- x 75-mm polypropylene tubes, #T1226-12 (American Scientific Products, McGaw Park, IL)
d. "FOAMRAC" sponge-rubber racks for decanting tubes (Bio-Rad Laboratories)
e. Sodium chloride (NaCl), ACS certified (Fisher Scientific Co.)

4. Reagent Preparation

a. Reconstitute the following materials as directed by Bio-Rad:
   (1) *"Lyphochek" levels I, II, and III*—Add 5.0 mL of distilled or deionized water. (Store at 2-8°C if not completely used on first day of analysis. Stable for 10 days.)

(2) *"Anemia control"*—Add 3 mL of distilled water. (Store at 4°C if not completed used on first day of analysis. Stable for 10 days.)

(3) *^{125}I-Antibody to ferritin tracer*—Add 11.0 ml distilled water slowly and carefully to tracer. Agitate gently to dissolve and allow to stand 5 min. (Stable at 2-8°C until expiration date.)

(4) *0.85 g/dL Sodium chloride*—Add 8.5 g sodium chloride to 300 mL distilled water in a 1-L volumetric flask. Mix well and dilute to volume. (Stable at 25°C; prepare weekly.)

(5) *Tracer/Immunobead Reagent*—Prepare this reagent immediately before beginning the assay from equal volumes of ^{125}I-ferritin antibody with ferritin Immunobeads. For each tube, 200 μL of combined reagent is required; prepare only the amount of combined reagent necessary for the run plus four extra tubes.

5. Standard Preparation

The ferritin standards are supplied in a stable liquid form, and no reconstitution is required. (Stable at 2-8°C until expiration date.)

6. Procedure

a. Sample preparation

Thaw frozen specimens completely and mix well. (Avoid repeated freezing and thawing of serum; this can cause erroneous results.)

b. Analysis

(1) Label two 12- x 75-mm polypropylene tubes for each standard, quality control pool, and each serum specimen, plus two total counts tubes.

(2) To the blank tubes, add 50 μL ferritin zero standard. To the standard tubes, add 50 μL of the appropriate standard—5.0, 10.0, 25.0, 100, 250, 1000, and 2500 ng/ml. Add 200 μL of the 2500 ng/ml to each of the maximum binding (MB) tubes. (The MB tubes will serve as B_0 in the assay.) Add 50 μL serum of each patient sample to the appropriate tubes.

(3) Mix Tracer/Immunobead Reagent thoroughly and add 200 μL to all tubes including the total counts tubes, using the Micromedic autodiluter. Set total counts tubes aside until counting time.

(4) Mix all tubes well and incubate in 37°C water bath for 1 h.

(5) Remove tubes from bath. Add 3 ml saline to all tubes and centrifuge for 10 min at 2000-3000 rpm to pack the beads at the bottom of the tubes.

(6) Immediately after centrifugation, insert tubes in FOAMRAC and invert to discard supernatant. (A large funnel is useful for collecting the supernatant and channeling it into a plastic bottle for proper disposal of the radioactive waste.) Blot tubes thoroughly on absorbent plastic-backed paper (and dispose of paper properly).

(7) Place tubes in racks for the gamma counter, and count each tube, including the total counts tube, for 1 min and record the counts.

7. Calculations

Both the LKB Rackgamma II and the Micromedic Apex have full data-reduction capabilities. Logit B/B_0 vs $\log_{10}$ concentration is used in both counters, where logit (B/B_0) = Ln $((B/B_0) / (1-B/B_0))$, B = corrected counts/min for each tube, and B_0 = maximum binding. In the IRMA, the zero standard is used for nonspecific binding and the maximum binding has been experimentally determined to be approximately four times the concentration of the 2500 ng/ml standard.

This method results in a linearized standard curve with a direct relationship of levels of radioactivity (counts per minute, or CPM) to concentration of ferritin in the sample. Serum results are expressed as nanograms of ferritin per milliliter of serum (ng/ml).

Performance checks for the assay include:

a. Zero standard

The CPM of the zero should be less than 6% of the CPM of the total counts.

b. High standard

The CPM of the 2500 ng/ml standard should be greater than 45% of the CPM of the total counts.

Serum samples assaying greater than 2500 ng/ml ferritin should be diluted 1:11 with the zero standard and reassayed, i.e., 50 μL serum diluted with 500 uL zero standard. Of this mixture, 50 μL is used for the assay. Samples with values less than 10 ng/ml should be reassayed for confirmation, as well as those samples whose duplicate results do not agree within 10%.

8. CDC Modifications

No CDC modifications were made to the standard protocol except to include the maximum binding tubes to permit automated data reduction with the logit-log function (as suggested by Bio-Rad Laboratories).

This kit was selected after an extensive evaluation of commercially available products. The accuracy of the kit was confirmed using materials kindly supplied by Dr. James Cook at the University of Kansas Medical School, Kansas City, KA, as well as the human liver ferritin international reference material supplied by the National Institute for Biological Standards and Controls, London, UK. Dr. Cook's laboratory also performed several comparison studies on our samples with IRMA and enzyme-linked immunoassay (ELISA) methods, and produced results very comparable to those from the HANES laboratory.

Section IV G References

1 Bio-Rad Laboratories. Instruction manual: Bulletin 4232: Quantimune Ferritin IRMA. Richmond, California: Bio-Rad Laboratories, June 1985.

2. Addison G, Hales C, Beamich M, et al. An immunoradiometric assay for ferritin in the serum of normal subjects and patients with iron deficiency and iron overload. J Clin Pathol 1972; 25:326-9.

3. Miles L In: Dekker M, Abraham GE, eds. Handbook of radioimmunassay. New York: Marcel Dekker, Inc, 1977.

V. QUALITY CONTROL

A. Preparation of Control Materials

For a long-term study such as HHANES, it is desirable to have stable quality control materials with different analytical concentrations, prepared in sufficient quantities to use throughout the survey. Serum pools used in the Division of Environmental Health Laboratory Sciences are prepared from pooled human serum (that has been screened for hepatitis). The serum is well mixed and stack- and sterile-filtered through 0.45- and 0.22-μ Millipore filters (Millipore Corporation, Bedford, MA) to remove cellular debris, detritus, and most bacteria. When low abnormal, normal, and elevated analyte levels of pools are to be prepared, the pooled serum is screened for concentrations of desired analyte(s), then divided into three portions. One portion is dispensed without modification as "normal," the second portion is diluted by 30% (v/v) with sterile 0.85 g/dL saline to achieve "low" concentration, and the third portion is concentrated by Amicon ultrafiltration (Amicon Corporation, Lexington, MA) or spiked with pure standard material of the desired analyte(s) to achieve "high" concentration. After the analyte levels are verified, sterile techniques are used under a laminar-flow hood in dispensing the serum, which is mixed while being aliquoted into sterile glass vials. The vials are sealed, labeled, and stored at -20°C or -70°C, depending upon the stability of the analyte(s) of interest.

The only pools not stored in glass vials are those for trace metals. Special precautions are taken in their preparation so that background contamination of elements such as copper and zinc will be minimal. The same high-density polyethylene vials used for specimen processing in HHANES were found to be acceptable as storage containers for trace metal pools because of their low contamination levels, designs that prevent leaking in the event of thawing, and tolerance to storage temperatures of -70°C.

Amber glass vials are valuable for the long-term protection of analytes such as vitamins A and E and protoporphyrin. Although these analytes are fairly stable stored at -20°C or lower, they are light-sensitive and may be degraded with prolonged light exposure (especially sunlight).

Whole blood pools for lead and protoporphyrin are collected from human donors, as well as from cows dosed with lead acetate to obtain elevated values. These pools are collected aseptically with 15% K_3EDTA as anticoagulant, screened for concentration levels, then mixed and dispensed under the same sterile conditions as serum pools.

Red cell folate hemolysate pools are not stable for more than 6 months and therefore are not useful for long-term studies. The same serum pools are used for both serum and red cell folate assays. Ascorbic acid is added (1 g/dL) to the serum to maintain the folate in a stable, reduced state, and the pools are lyophilized to gain maximum stability.

Glucose pools are prepared from serum that has been spiked with pure D-glucose, with a final concentration of 0.1 g/dL benzoic acid added as a preservative.

B. System Description

Quality control is the system with which the laboratorian determines that a method is acceptable by measuring the variability in an analytical process. The results of these measurements are evaluated against predetermined statistical criteria and specifications.

Each analytical procedure described in this manual was chosen on the basis of methodological evaluations to determine its inherent accuracy, precision, and reliability for a long-term study. "State of the art" instruments were used as much as practical. Some methods, like glucose, were direct adaptations of the reference method; others, such as folate, were commercially available kits selected after extensive evaluations and used under standardized conditions. Primary standards or reference materials such as National Bureau of Standards Standard Reference Materials ("NBS SRM's") were used when available to validate analytical accuracy. Estimates of imprecision were generated from long-term quality control pool results. To create a bridge of continuity with

NHANES II (1976-1980), certain stable quality control pools were used for methods such as iron, TIBC, and folate for the latter part of NHANES II and the first half of HHANES (*1*).

Two types of quality control systems are used for biochemical analyses in the HANES laboratory. These two systems are: (1) "bench" quality control pools inserted by the analyst and measured from two to four times in each analytical "run" (a set of consecutive assays performed without interruption) so that judgments may be made on the day of analysis and (2) "blind" quality control samples that are placed in vials, labeled, and processed so that they are indistinguishable from regular HHANES samples. The results from blind specimens are decoded and reviewed by the supervisor. With both systems, all levels of analyte concentration are assessed by taking these samples through the complete analytical process. The data from these materials are then used in estimating methodological imprecision and in assessing the magnitude of any time-associated trends.

For each analyte, two levels of blind quality control pools are used. These pools are prepared in sufficient quantity to last throughout the survey. The levels chosen are generally in the "low normal" and "high normal" ranges so as not to be obvious to the analyst. The pools are prepared in the same manner as bench pools for each analyte, but they are dispensed in vials identical to those used in the field for HHANES samples, labeled with pseudoparticipant numbers corresponding to each geographical location of the survey, and stored at -20°C. At least one blind sample is randomly incorporated with every 20 HHANES samples and analyzed singly or in duplicate, according to the assay protocol.

As previously mentioned, bench quality control pools comprise three or more levels of concentration spanning the low, normal, and elevated ranges for each analyte (except glucose and lead, which are analyzed in different laboratories).

Quality control limits are established for each pool. Either a one-way or a two-way classification analysis of variance (ANOVA) (*2*) is performed, depending upon the run format of the analysis.

For "batch" methods such as protoporphyrin or folate, all standards, control pools, and specimens are subjected to the same treatment simultaneously, e.g., extraction or precipitation steps. Usually only one large run is performed per day. At least two measurements are made of three levels of bench pools.

For batch methods, the one-way analysis of variance is used. In this case a day is equal to a run, and only one vial of each pool is used. Time in days is designated as the classification variable so that within-day and among-day components of variance may be estimated. The total variance is then the sum of within- and among-day components.

For other analyses such as iron and TIBC (run as "AM" and "PM" modes, with each half of the day treated independently), or lead (run with bracketed sets of standards), the two-way nested analysis of variance is used. In these assays, multiple runs occur within a day (and multiple vials of each pool are used). This allows computation of the additional among-run (or among-vials), within-day component.

Additional information about the Division of Environmental Health Laboratory Sciences statistical quality control system is available in the laboratory manual for NHANES I (1971-1975) (*3*).

Section V B References

1. Gunter EW, Turner WE, Neese JW, Bayse DD. Laboratory procedures used by the Clinical Chemistry Division, Centers for Disease Control, for the Second Health and Nutrition Examination Survey (HANES II) 1976-1980. Atlanta: Centers for Disease Control, 1981 50-68.
2. Ostle B. Statistics in research. 2nd ed. Ames, Iowa: Iowa State University Press, 1963.
3. National Center for Health Statistics HANES I: Hematology and clinical chemistry procedures developed or utilized by the Center for Disease Control, Bureau of Laboratories, 1971-1975. Part 16. Washington, D.C. U.S. Government Printing Office, Aug 1979:79-93.

C. Analysis of Variance Tables and Long-Term Quality Control Charts

Tables 4-12 and Figures 2-11 provide quality control data on specific analytes.

TABLE 4.
HISPANIC HANES QUALITY CONTROL SUMMARY: ERYTHROCYTE PROTOPORPHYRIN

POOL NO.	DATES	MEAN (μg/dL RBC)	STANDARD DEVIATION (μg/dL RBC)	COEFFICIENT OF VARIATION (%)	NUMBER OF OBSERVATIONS
2681	8/30/83-8/18/83	51.138	2.38	4.66	364
2781	8/30/82-8/18/83	113.809	6.57	5.77	366
2881	8/30/82-8/18/83	163.919	7.65	4.67	368
1882	8/30/82-6/30/83	27.785	1.58	5.48	268
2683	7/13/83-1/8/85	51.637	1.86	3.61	399
2783	7/13/83-1/8/85	115.816	4.93	4.25	391
2883	7/13/83-1/8/85	166.322	6.02	3.62	400
1883	7/13/83-1/8/85	35.734	1.35	3.78	392

FIGURE 2.

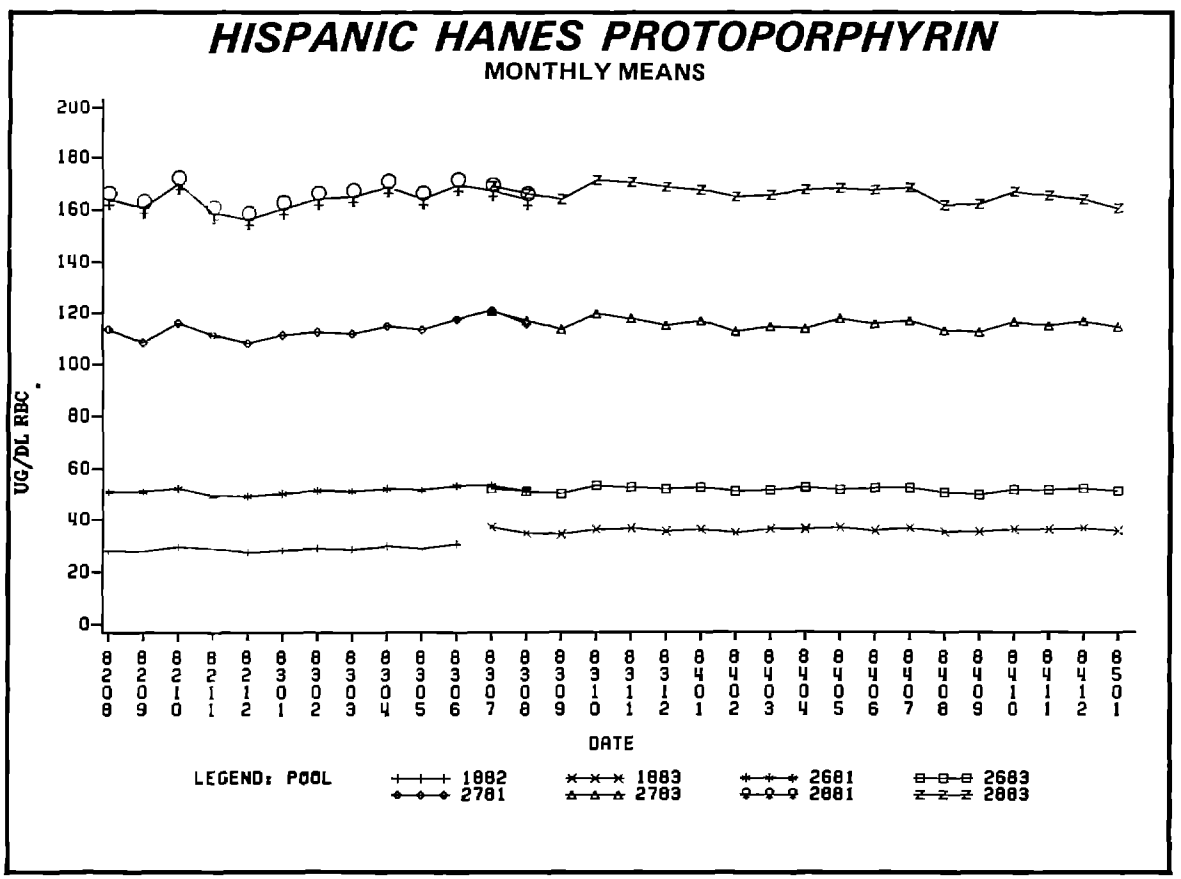


TABLE 5.
HISPANIC HANES QUALITY CONTROL SUMMARY: SERUM IRON

POOL NO.	DATES	MEAN (μg/dL)	STANDARD DEVIATION (μg/dL)	COEFFICIENT OF VARIATION (%)	NUMBER OF OBSERVATIONS
1679	9/14/82-5/25/83	76.663	1.91	2.50	336
1779	9/14/82-5/25/83	101.429	2.12	2.10	311
1879	9/14/82-4/05/83	163.466	2.82	1.72	261
882	2/28/83-1/22/85	79.663	1.60	2.00	792
982	2/28/83-1/22/85	98.100	1.86	1.89	787
1082	2/28/83-1/22/85	136.770	2.23	1.06	782

FIGURE 3.

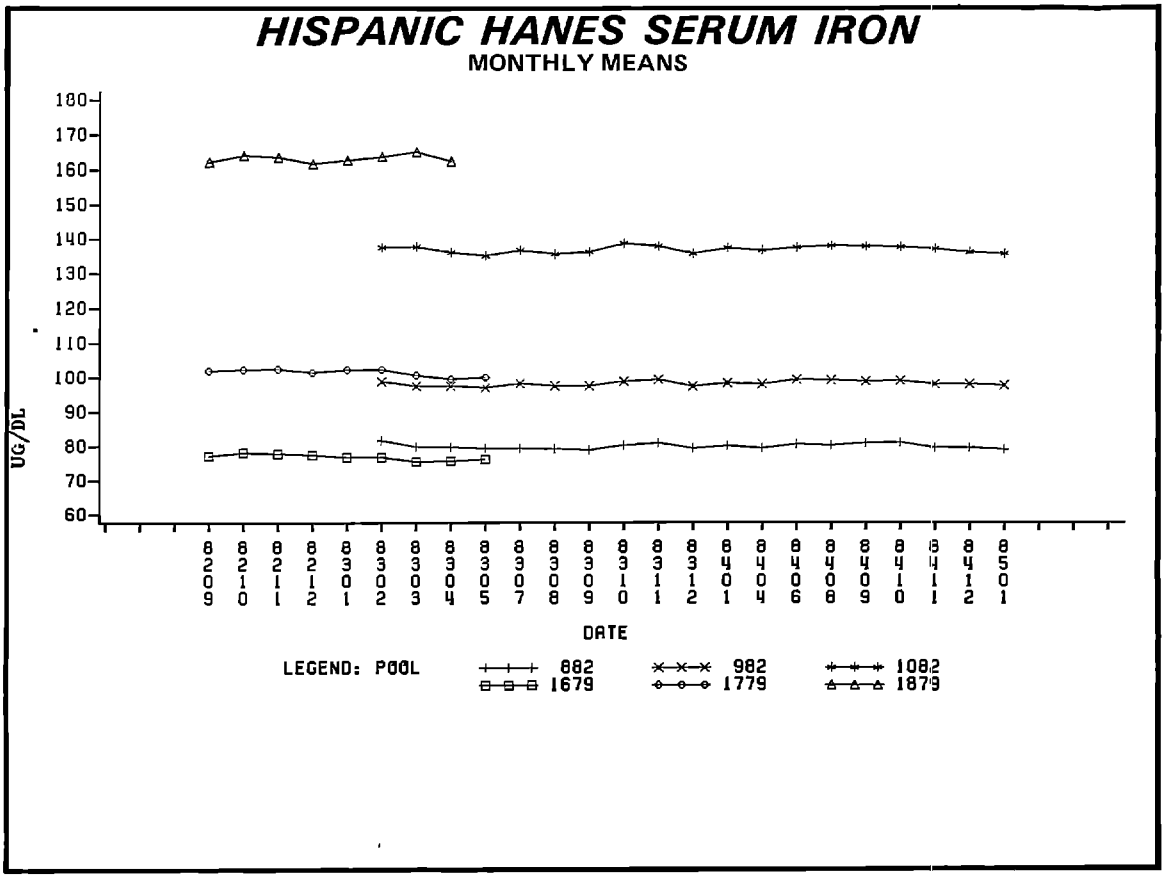


TABLE 6.
HISPANIC HANES QUALITY CONTROL SUMMARY: SERUM TOTAL IRON-BINDING CAPACITY

POOL NO.	DATES	MEAN (μg/dL)	STANDARD DEVIATION (μg/dL)	COEFFICIENT OF VARIATION (%)	NUMBER OF OBSERVATIONS
1679	10/7/82-6/9/83	255.963	5.41	2.11	240
1779	10/7/82-6/9/83	372.582	6.28	1.69	234
1879	10/7/82-4/14/83	574.300	10.14	1.77	225
882	3/16/83-3/15/85	312.390	5.86	1.88	853
982	3/16/83-3/15/85	392.792	7.34	1.87	862
1082	3/16/83-3/15/85	551.914	8.09	1.47	848

FIGURE 4.

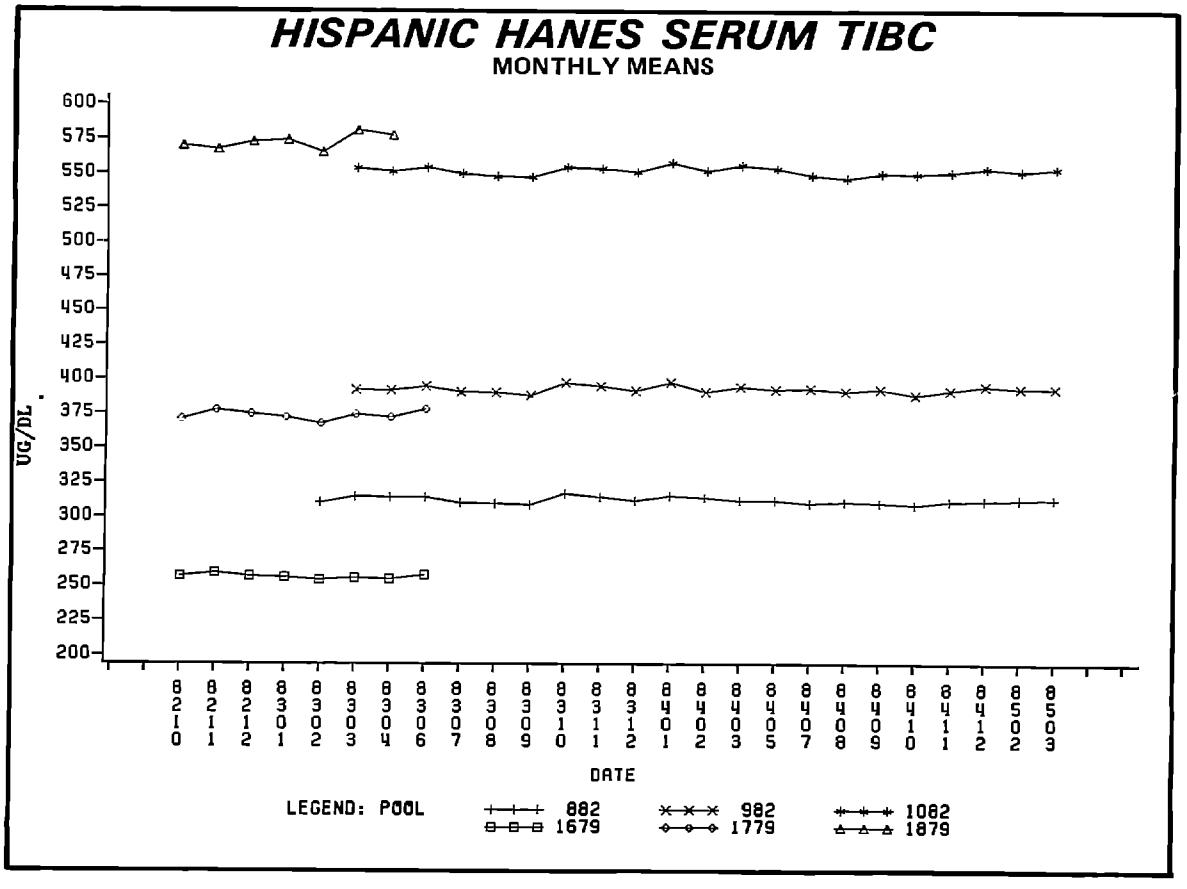


TABLE 7.
HISPANIC HANES QUALITY CONTROL SUMMARY: VITAMIN A

POOL NO.	DATES	MEAN (μg/dL)	STANDARD DEVIATION (μg/dL)	COEFFICIENT OF VARIATION (%)	NUMBER OF OBSERVATIONS
5821	9/21/82-3/28/83	27.507	1.81	6.58	151
6821	9/21/82-3/28/83	45.911	2.22	4.83	153
7821	9/21/82-3/28/83	71.927	3.54	4.92	149
5823	6/1/83-6/7/84	27.578	2.41	8.75	273
6823	6/1/83-3/12/84	47.547	3.33	7.00	216
7823	6/1/83-8/1/84	74.879	4.41	5.89	274
184	1/31/84-2/5/85	43.313	2.76	6.38	201
284	3/21/84-2/5/85	23.946	2.25	9.40	181
384	3/21/84-2/5/85	73.434	4.59	4.59	189

FIGURE 5.

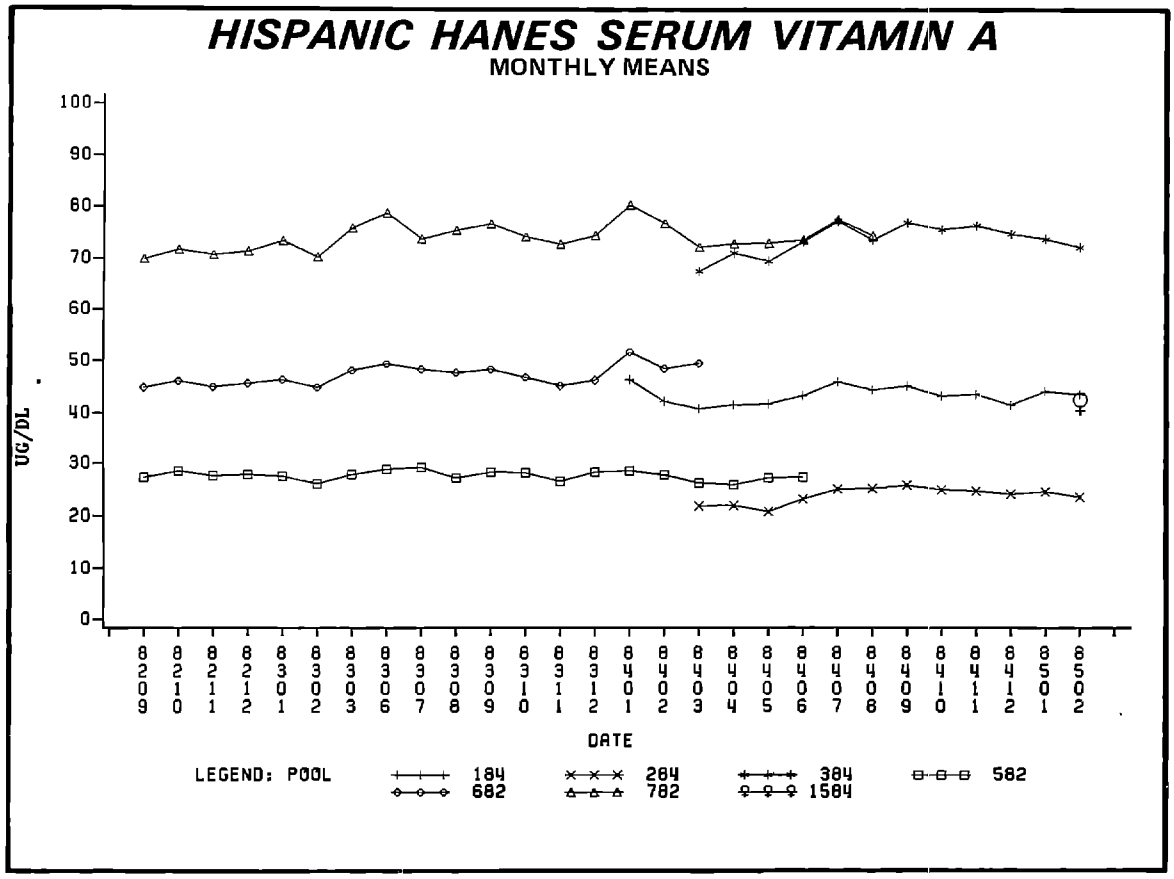


TABLE 8.
HISPANIC HANES QUALITY CONTROL SUMMARY: VITAMIN E

POOL NO.	DATES	MEAN (μg/dL)	STANDARD DEVIATION (μg/dL)	COEFFICIENT OF VARIATION (%)	NUMBER OF OBSERVATIONS
5821	9/21/82-3/28/83	773.59	74.30	9.61	151
6821	9/21/82-3/28/83	1413.56	121.92	8.63	151
7821	9/21/82-3/28/83	2032.59	166.96	8.21	155
5823	6/1/83-6/7/84	719.08	68.21	9.49	282
6823	6/1/83-3/12/84	1394.52	112.33	8.06	232
7823	6/1/83-8/1/84	1996.89	166.46	8.34	294
184	1/31/84-2/5/85	1165.09	88.89	7.62	197
284	3/21/84-2/5/85	494.68	52.63	10.64	173
384	3/21/84-2/5/85	1654.99	190.66	11.52	187

FIGURE 6.

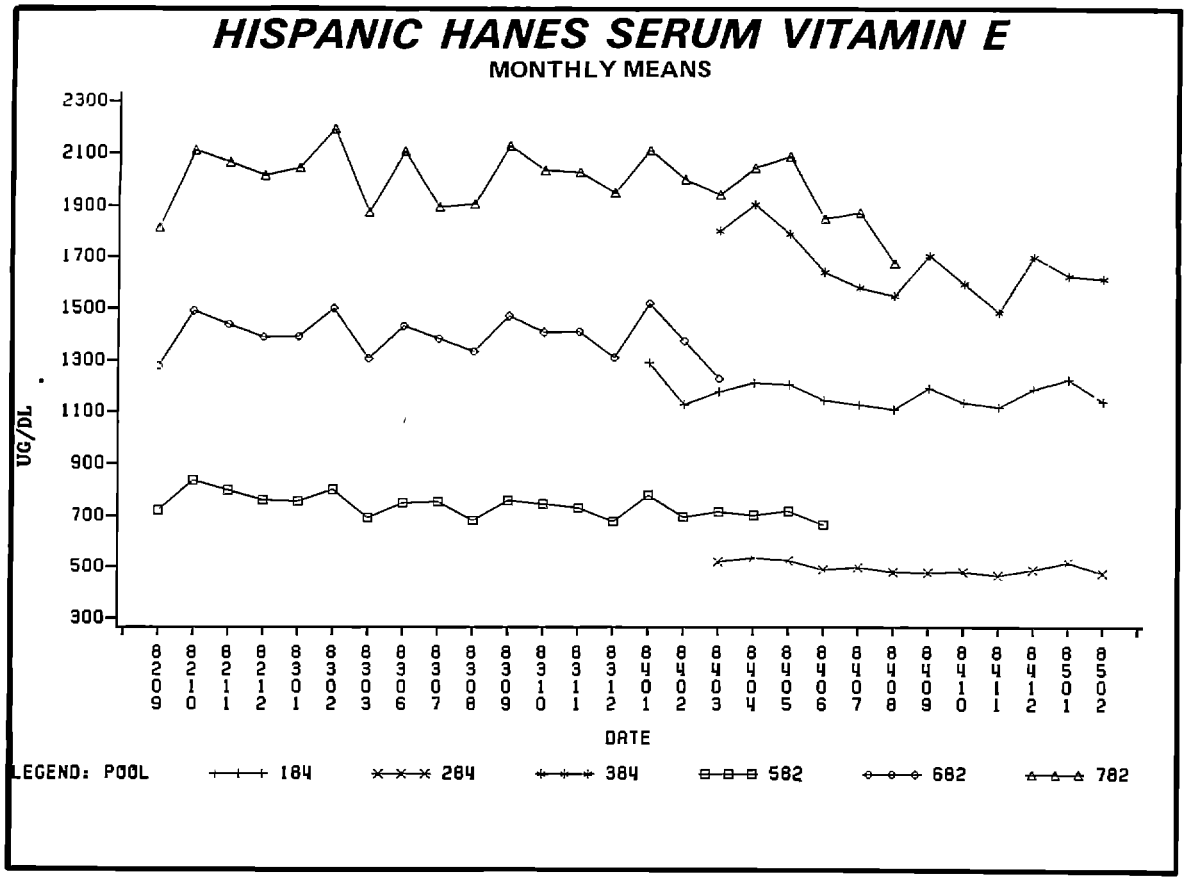


TABLE 9.
HISPANIC HANES QUALITY CONTROL SUMMARY: GLUCOSE

POOL NO.	DATES	MEAN (μg/dL)	STANDARD DEVIATION (μg/dL)	COEFFICIENT OF VARIATION (%)	NUMBER OF OBSERVATIONS
2181	11/18/82-2/5/85	93.507	1.74	1.86	277
4679	11/18/82-2/5/85	126.633	1.56	1.23	266

FIGURE 7.

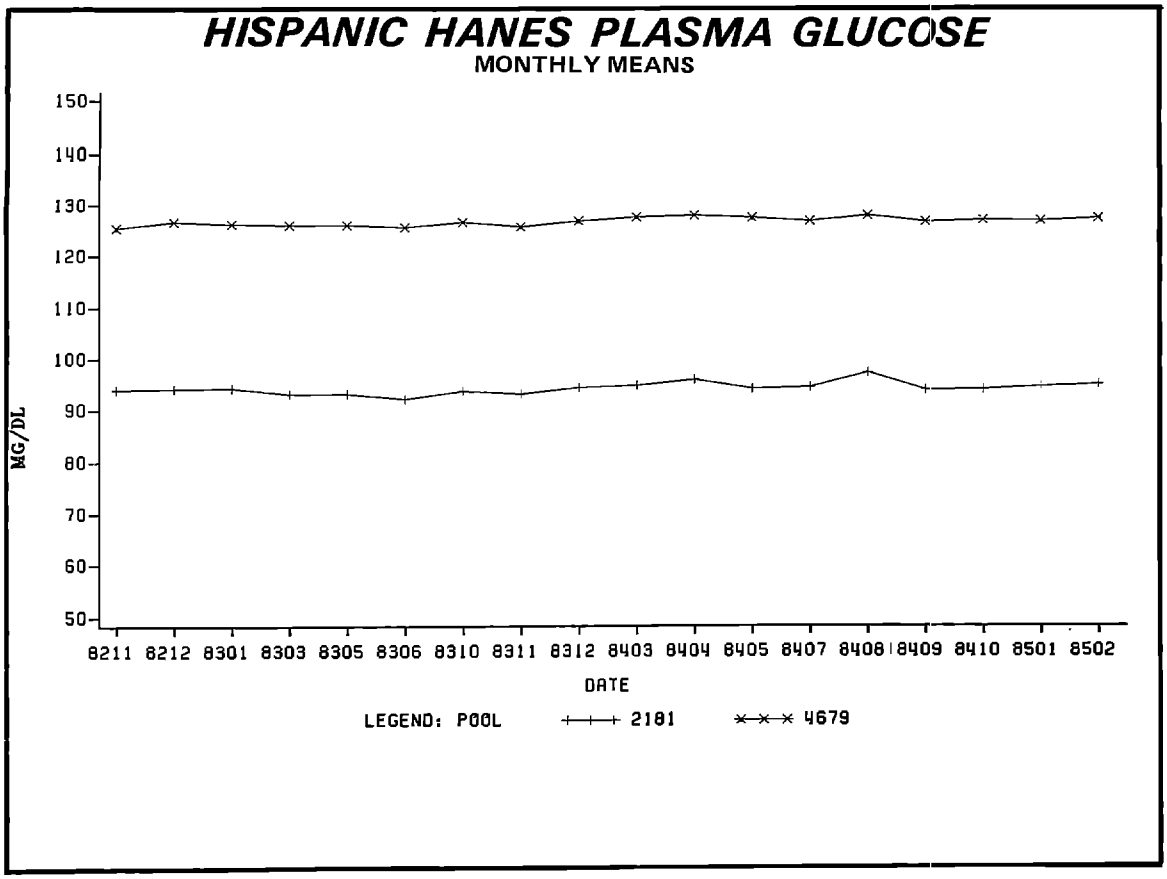


TABLE 10.
HISPANIC HANES QUALITY CONTROL SUMMARY: RED CELL FOLATE

POOL NO.	DATES	MEAN (μg/dL)	STANDARD DEVIATION (μg/dL)	COEFFICIENT OF VARIATION (%)	NUMBER OF OBSERVATIONS
4877	10/21/82-10/26/83	3.526	0.21	5.98	74
4977	10/21/82-1/19/84	8.080	0.50	6.18	114
5077	10/21/82-5/12/83	11.797	0.60	5.11	80
6077	10/21/82-11/10/83	4.152	0.33	8.02	101
2383	10/26/83-2/22/85	4.709	0.24	5.19	101
2483	2/21/84-2/22/85	5.061	0.25	5.04	87
2583	10/26/83-2/22/85	8.762	0.56	6.38	108
30183	12/13/83-2/22/85	1.341	0.15	11.15	102

FIGURE 8.

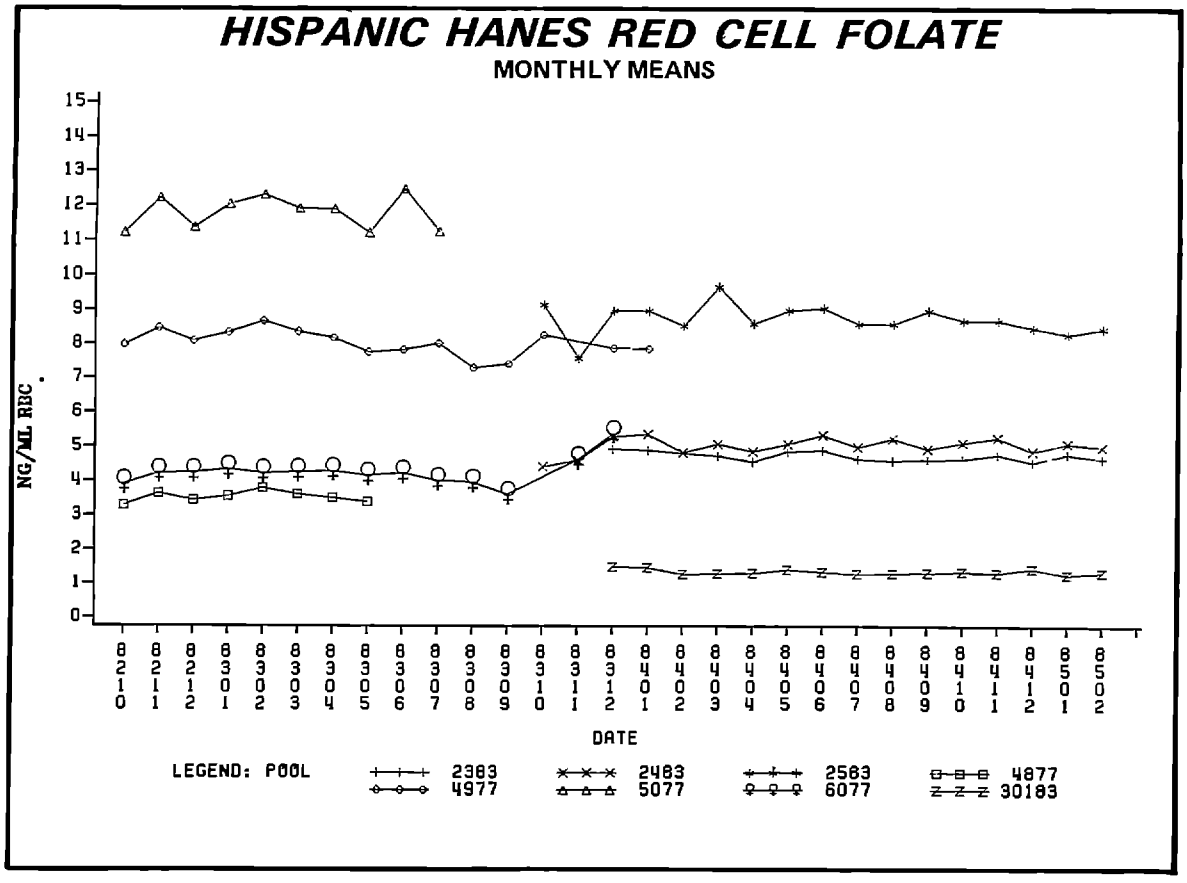


TABLE 11.
HISPANIC HANES QUALITY CONTROL SUMMARY: SERUM FOLATE

POOL NO.	DATES	MEAN (μg/dL)	STANDARD DEVIATION (μg/dL)	COEFFICIENT OF VARIATION (%)	NUMBER OF OBSERVATIONS
4877	10/7/82-5/5/83	3.570	0.17	4.86	76
4977	10/7/82-2/21/84	7.944	0.50	6.26	130
5077	10/7/82-5/5/83	11.802	0.61	5.19	80
6077	10/7/82-12/5/83	4.114	0.26	6.34	110
2383	10/12/83-2/13/85	4.687	0.21	4.52	102
2483	2/21/84-2/13/85	5.051	0.24	0.67	86
2583	10/12/83-2/13/85	8.689	0.46	5.29	104
30183	12/19/83-2/13/85	1.363	0.15	10.97	96

FIGURE 9.

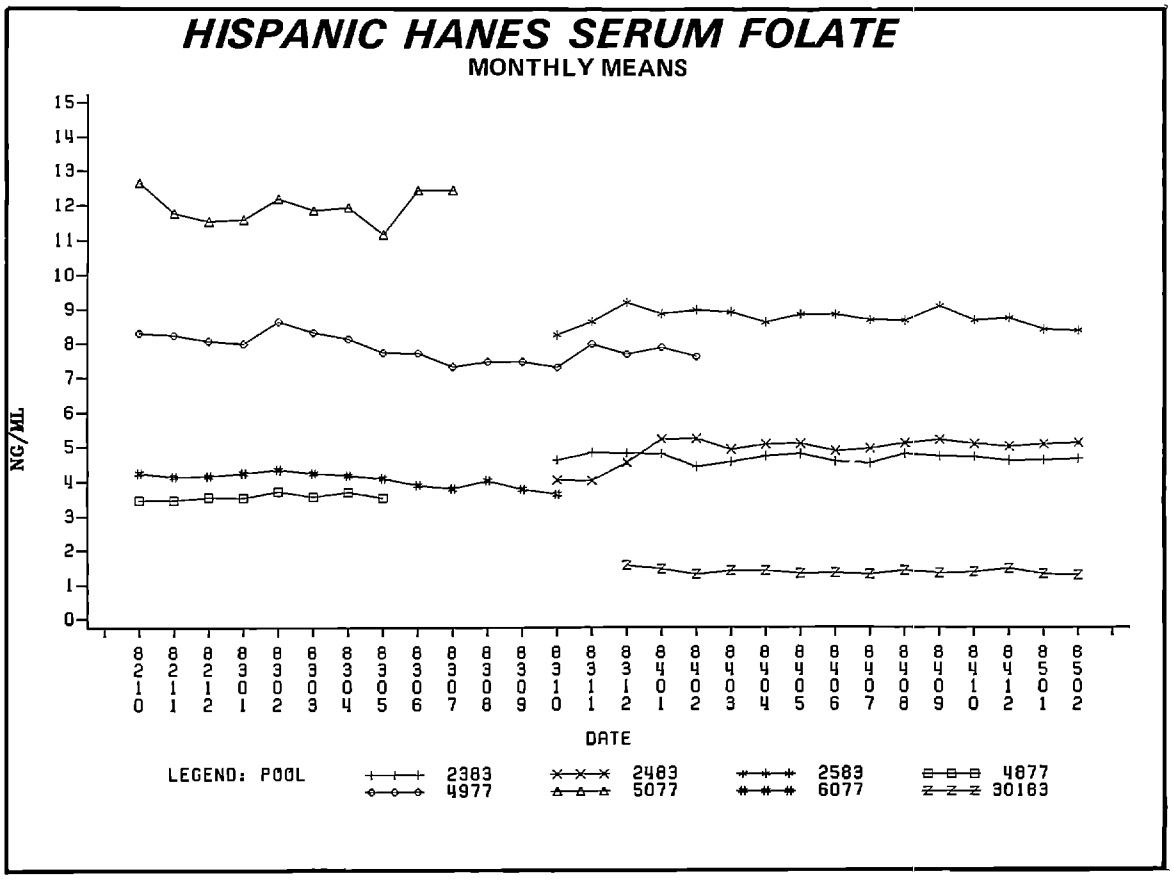


TABLE 12.
HISPANIC HANES QUALITY CONTROL SUMMARY: BLOOD LEAD

POOL NO.	DATES	MEAN (μg/dL)	STANDARD DEVIATION (μg/dL)	COEFFICIENT OF VARIATION (%)	NUMBER OF OBSERVATIONS
2082	10/18/82-6/5/85	12.736	1.73	13.61	328
2182	10/18/82-6/5/85	31.022	3.44	11.13	328

FIGURE 10.

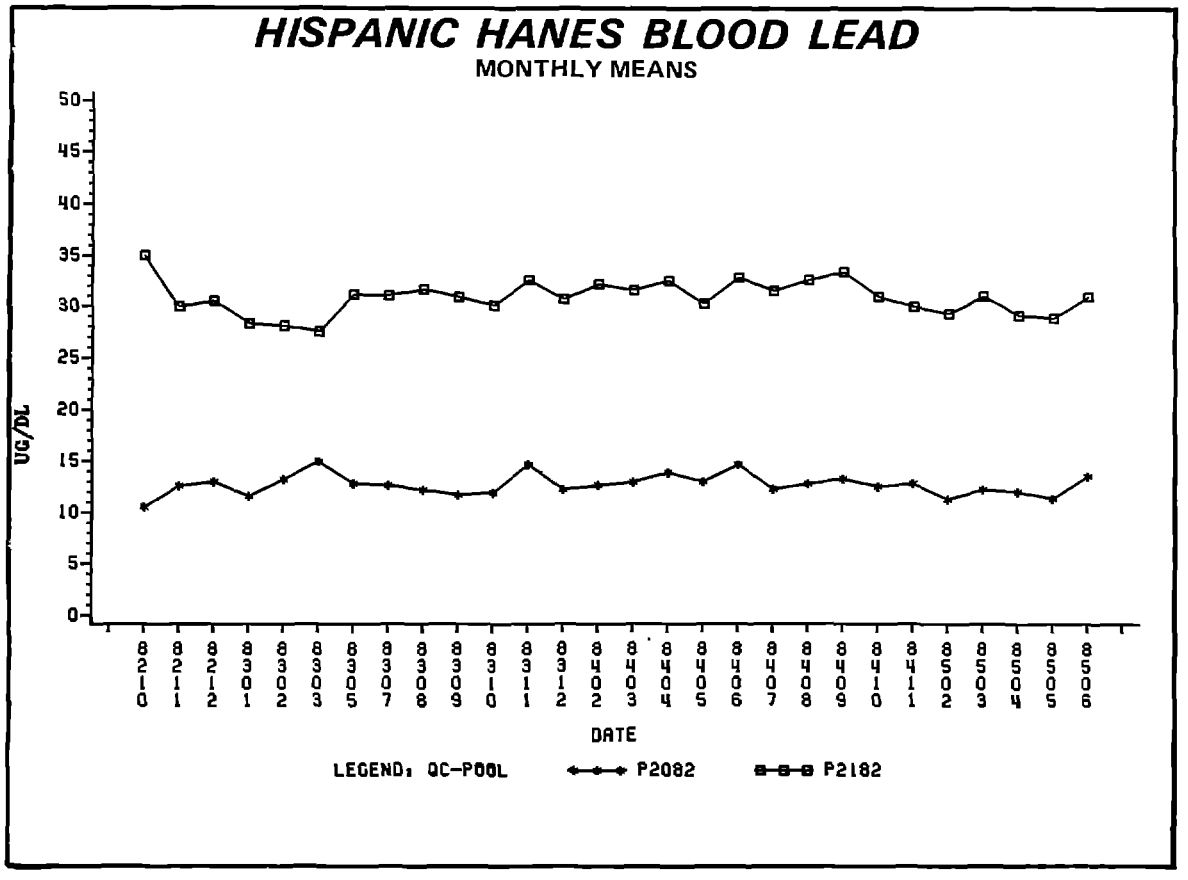


TABLE 13.
HISPANIC HANES QUALITY CONTROL SUMMARY: SERUM FERRITIN

POOL NO.	DATES	MEAN (ηg/mL)	STANDARD DEVIATION (ηg/mL)	COEFFICIENT OF VARIATION (%)	NUMBER OF OBSERVATIONS
1384	11/5/84-3/28/85	38.206	2.71	7.11	84
1484	11/5/84-3/28/85	140.876	25.90	18.43	88
1584	11/5/84-3/28/85	422.932	48.82	11.52	90
1684	11/5/84-3/28/85	5.592	1.13	20.24	88
2384	3/29/85-1/3/86	32.492	3.07	9.44	210
2484	3/29/85-1/3/86	137.686	9.97	7.24	210
2584	3/29/85-1/3/86	376.164	28.58	7.60	212
2684	3/29/85-1/3/86	4.402	1.08	24.55	203
884	11/13/84-1/3/86	52.015	5.30	10.18	294
6685	7/25/85-12/10/85	3.318	0.87	26.09	136

FIGURE 11.

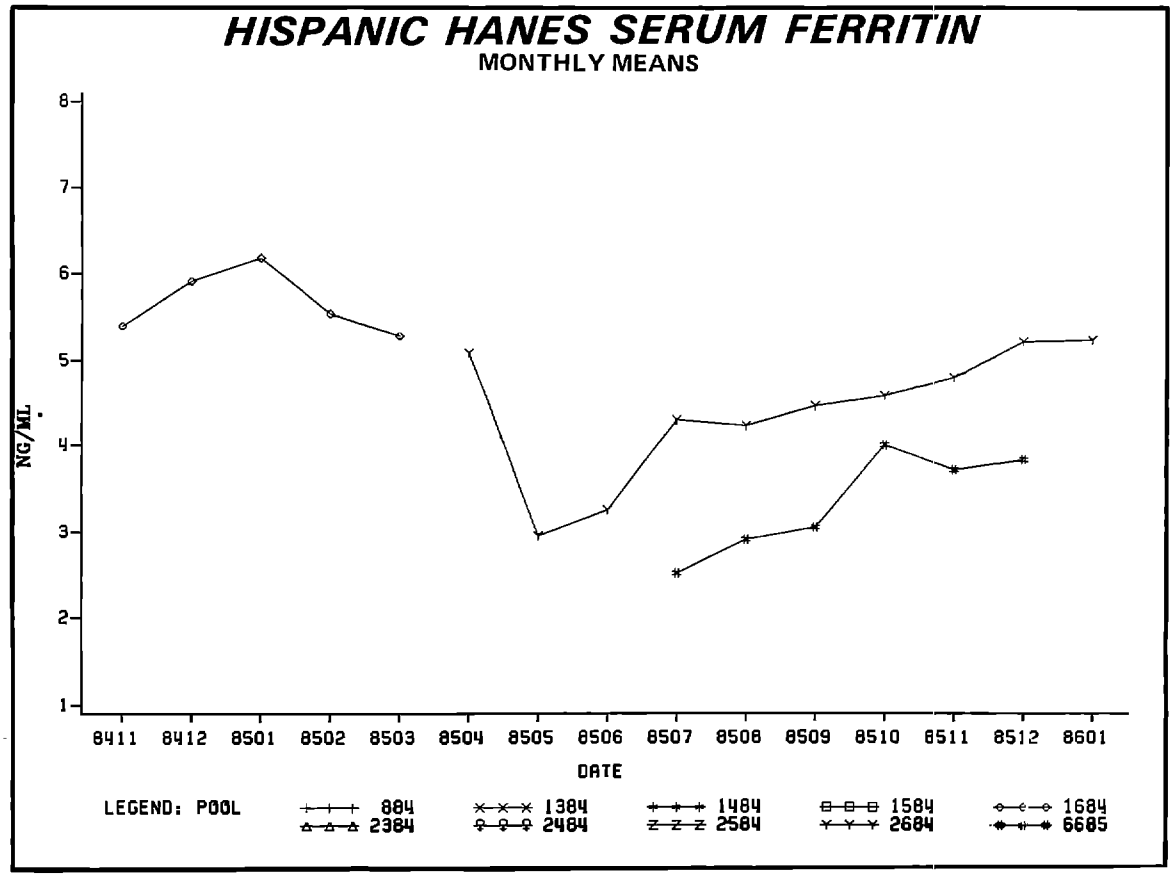


FIGURE 12.

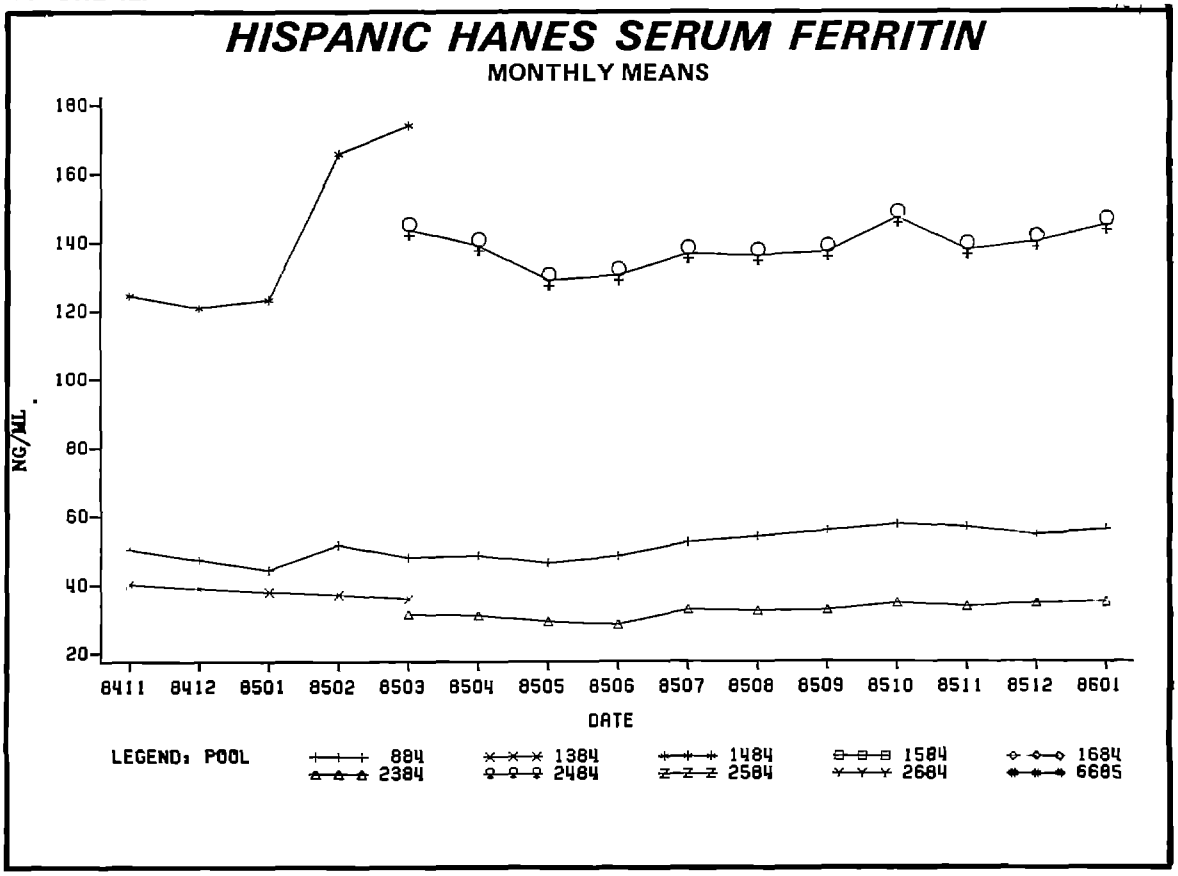
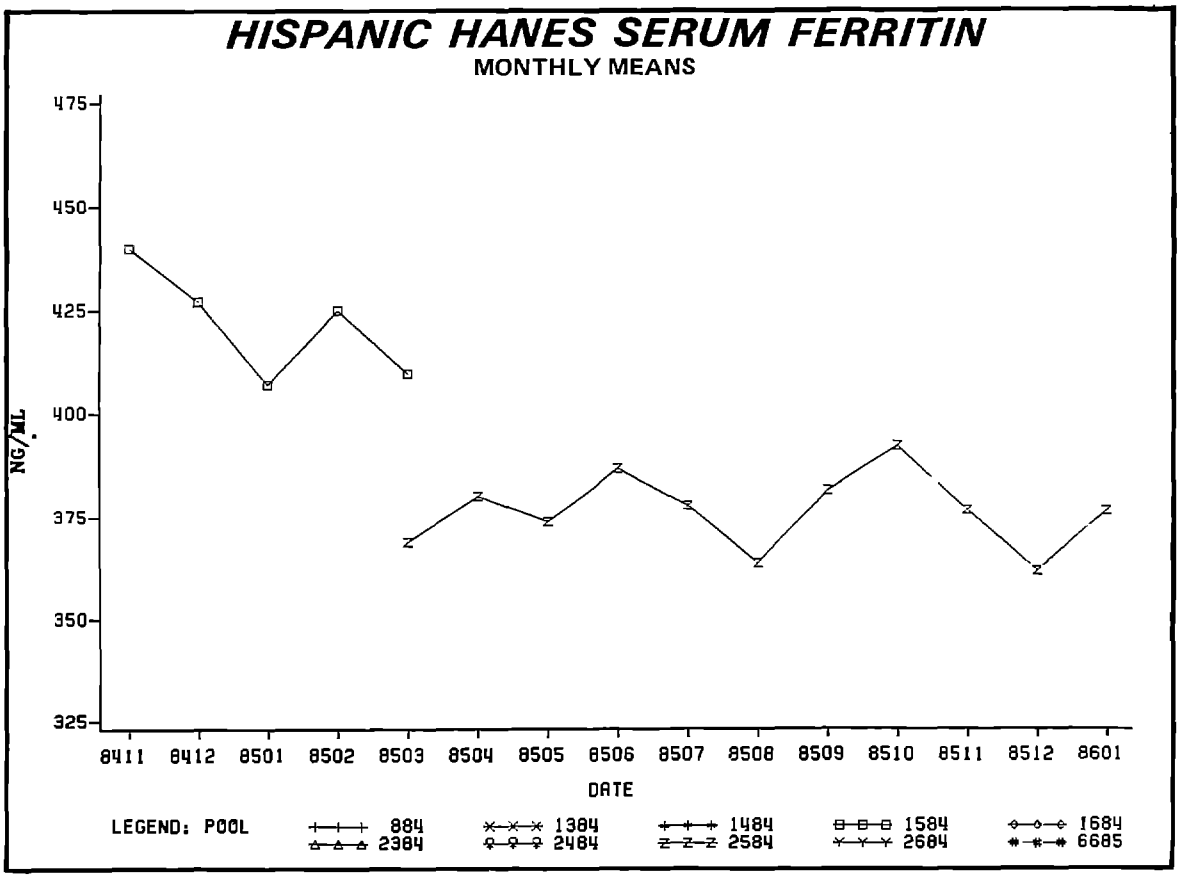


FIGURE 13.



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