NBSIR 75-[illegible]

Final Report
FDA Contract No. 74-82(0)

NBSIR 79-1797

FINAL REPORT
FDA CONTRACT NO. 74-[illegible]

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National Bureau of Standards
Washington, D.C. 20234

August 1979

Report for Period
October 1977 to [illegible] 1978
Issued August 1979

Prepared for
Bureau of Medical Devices and Diagnostic Products
Food and Drug Administration
Rockville, Maryland 20[illegible]

U.S. DEPARTMENT OF COMMERCE [illegible], Secretary

Luther H. [illegible], Jr., Under Secretary

Jordan J. Baruch, Assistant Secretary for [illegible]

NATIONAL BUREAU OF STANDARDS, [illegible], Director

Report on FDA Contract No. 74-58(.)

for the period

October 1977 to September 1978

Issued August 1979

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Prepared for

Bureau of Medical Devices and
Diagnostic Products
Food and Drug Administration
Rockville, Maryland 20852

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INTRODUCTION

This report describes work performed at the National Bureau of Standards from October 1971 through September 1973 in the continuation of project initiated under NBS Report 74-58(0). Some of the early results were already mentioned but not given in detail in the previous report in this series which was written after September 1972.

Task 1a. GLUCOSE IN SERUM

A. Glucose in the WHO Reference Serum

Six vials of frozen serum, selected randomly among all the vials were thawed and pooled. Eight samples were taken from this pool and weighed. Four were treated with weighed portions of an aqueous glucose-U-^{13}C solution of known concentration. The other four were treated similarly but with a separately prepared, known solution of the labeled glucose. After mixing and allowing approximately three hours at room temperature for reaching equilibrium, the isotope-enriched samples were freeze-dried and then were treated to convert the glucose into 1,2:3,6 di-O isopropylidene D-glucose. The latter was isolated by sublimation and then purified by TLC to remove the possibility of contamination by other di-O-isopropylidene hexoses.

GC/MS was performed by injecting chloroform solutions of purified glucose derivative from the samples and from calibration mixtures into a column containing three percent OV-17 on 100/120 mesh Gas Chrom Q and monitoring the intensity ratio of labeled to unlabeled $(M-15)^+$ ions at m/e 250 and 245, respectively, in a medium resolution mass spectrometer that employs magnetic field switching and is equipped for recording the signals due to the selected ions.

Measurements on each sample were made in duplicate on each of two days. Sample concentrations were calculated by linear interpolation of the intensity ratios given by the

calibration mixtures that closely bracketed the isotopic composition of that sample and that were run in duplicate immediately before and after each sample. The individual values, given in Table 1, are averages of the daily duplicate measurements.

Task 1a, Table 1. Corrected Glucose Levels in the NBS Reference Serum (mg/g)

Sample Number	Day 1	Day 2
1	982.6	981.1
2	980.5	980.3
3	982.3	981.3
4	983.4	981.3
5	979.7	980.2
6	978.0	980.8
7	979.5	978.4
8	979.4	978.8

mean = 980.6 mg/L
= 5.444 mmol/L
RSD = 0.19%

If the mean of the values for the first four (day 1) compared to the mean of the second four, a 1.9 mg/L (0.01 mmol/L) difference is observed. Since the samples were taken from a common pool, the same calibration mixtures were used for all analyses, and the values obtained fall in rather distinct groups

the solutions of the glucose-U-^{14}C appear to be the source of the systematic difference. This could be due to an error in the calculated concentrations of the two glucose-U-^{14}C solutions that might have arisen from weighing errors or an impurity in the glucose-U-^{13}C, or both. Such concentration errors would be transferred to the samples and lead to the differences found.

The glucose concentrations given in Table 1 are corrected values, i.e., not those originally reported to the WHO. Correction was found necessary when the series of calibration mixtures employed in their measurement were tested for consistency. The series was composed of two smaller interlocked series. The highest and the lowest calibration mixtures came from only one of the series. This consistency test was done by bracketing each calibration mixture in turn with the pair of calibration mixtures having a higher and a lower weight-ratio than that of the intermediate mixture, and calculating the weight-ratio of the intermediate mixture from the measured ion-intensity ratios of the three mixtures, assuming the weight-ratios of the outer pair to be correct. One of the calibration mixtures was found to be in error, necessitating its correction, and then the correction of the values for the WHO reference pool. The correction amounted to a lowering of 0.14 percent.

The WHO reference pool measurements demonstrate more than our mass spectrometric measurement protocol was capable of such high precision, but some of the systematic errors were

being made evident and soon to be corrected. Even without correction, the precision of the results is sufficiently high for acceptability in a definitive method.

B. Analyses of Human Serum Pools

As described in our previous report under Glucose in Serum item C, these new serum pools were analyzed to examine the time-dependence of their glucose concentrations as well as to verify the concentration-dependence of the bias between the definitive and reference methods. A plot of the [illegible] results, showing the decline in the glucose concentrations of all the serum pools with time, was shown in our previous report. The data are presented here in Task 1a, Table 2.

Samples of these pools were also analyzed by the reference method at the CDC. The period of time over which the CDC analyzed these pools was longer than the period through which definitive method analyses were run at NBS. The CDC results showed that over the 18-month period the pool concentrations fell between 2.6 and 3.9 percent.

A comparison of the data for a two month period of time during which both methods were being performed is shown in Task 1a, Table 3. Reference method values are consistently lower than the definitive method values, the bias being greatest at the highest concentration levels.

Task 1a, Table 7. Glucose Levels (mg/dL) in [illegible]

Analysis Starting Date	Measurement Day	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
10/20/77	1		1[illegible].6	1[illegible]	[illegible]		[illegible]
	2		1177.7	36[illegible].6	2[illegible]		3[illegible]
11/15/77	1	670.4	1181.2	1698.2	[illegible]	2[illegible].5	[illegible]
	2	6[illegible].7	1181.2	16[illegible]	21[illegible]	[illegible]	[illegible]
12/6/77	1	667.6	1180.8	1691.0	2196.[illegible]	[illegible]	[illegible]
	2	668.2	117[illegible]6	16[illegible].0	21[illegible]	[illegible]	[illegible]
1/17/78	1	[illegible]6.2	[illegible]10.[illegible]	16[illegible].0	2192.[illegible]	28[illegible]	[illegible]
	2	668.2	1169.7	1661.[illegible]	2135.[illegible]	28[illegible]	[illegible]
3/27/78	1	[illegible]6[illegible].0	1175.8	1680.0	2170.[illegible]	2851.4	[illegible]
	2	664.6	1172.9	1672.[illegible]0	2177.6	2853.0	[illegible]
4/18/78	1	663.4	1164.7	1672.2	2165.8	2835.7	[illegible]6
	2	661.7	1161.4	1668.4	2167.[illegible]	[illegible].2	[illegible]

Task 10, Table 7. Comparison of the Definitive (DM) and Reference (RM) Methods for Glucose [illegible] Human Serum Pool

Pool	DM Mean[1] mg/L	RM Mean[2] mg/L	DM-RM mg/L	$\frac{DM-RM}{DM}$ x 100 Percent
3077	661.5	665.4	[illegible]	0.58
3177	117[illegible]	116[illegible]	7.8	[illegible]
3277	16[illegible]	166[illegible]	16.8	0.[illegible]
3377	21[illegible]	[illegible]	[illegible]	[illegible]
3477	26[illegible]	[illegible]	[illegible]	[illegible]
3577	36[illegible]	361[illegible]	[illegible]	[illegible]

Pool	DM 12/?/77 mg/L	RM 12/5/77 mg/L	DM-RM mg/L	$\frac{DM-RM}{DM}$ x 100 Percent
3077	66[illegible]	[illegible]	7.1	[illegible]
3177	118[illegible]	117[illegible]	[illegible]	0.75
3277	16[illegible]	1672.1	10.2	0.64
3377	21[illegible]	2171.7	[illegible]	1.0[illegible]
3477	26[illegible]	25[illegible]	[illegible]	[illegible]
3577	36[illegible]	3629.1	[illegible]	[illegible]

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[1] Mean value by the definitive method for analyses done December 6, 1977, and January 17, 1978.

[2] Mean value by the reference method for [illegible] over the period December 5, 1977, through February 15, 1978 [illegible] for each pool.

Task 1a, Table 3. (Continued)

Pool	DM 1/17/78 mg/L	RM 1/5 & 25/78 mg/L	Diff. mg/L	Diff. %
3077	666.0	665.1	0.9	0.1
3177	1171.0	1166.8	4.2	0.3
3277	1681.7	1685.9	15.2	0.99
3377	2184.1	2154.2	29.9	1.37
3477	2857.0	2821.8	33.2	1.1
3577	3659.4	3610.4	49.1	1.3

C. An Alternative Definitive Method for Glucose

As indicated in our previous report, we had begun research on another definitive method that could be used in parallel with the original definitive method and thereby demonstrate long term definitive method stability, for which evidence is presently lacking. (That the CDC had also found evidence for the long term instability of the [illegible] was unknown to us until recently.)

For the alternative definitive method a new supply of glucose-U-^{13}C is being used. It has a higher ^{13}C-enrichment and glucose labelled with six ^{13}C atoms is present in about a 50-percent abundance. After derivatization with butaneboronic acid the most abundant mass is five mass units greater than for ordinary glucose because of the loss of one ^{13}C. The method involves the following: About 1 mg of the glucose-U-^{13}C is added to a serum aliquot that contains about 0.5 mg of glucose. After time is allowed for the labeled and unlabeled glucose to equilibrate, ethyl alcohol is added to precipitate protein. The supernatant is freed of ethyl alcohol and then deionized using an ion-exchange resin. The solution is freeze-dried, the residue is treated with pyridine and butaneboronic acid, and the mixture is heated at 60°C for 0.5 hour. Finally, the mixture is treated with acetic anhydride for one hour and then is evaporated to dryness. The residue is dissolved in isooctane for GC/MS. (Compared to the original definitive method, the present one appears to require much less effort to prepare samples for GC/MS.)

GC/MS is performed by injecting aliquots of solution which contain the bis(butaneboronate) acetate derivative of glucose onto a capillary gas chromatographic column where the glucose derivative is completely resolved from derivatives of similar structure that would be formed if fructose, galactose, or mannose were present in the urine sample.

For each analysis, aliquots of a low or high dilution mixture are injected before and after the sample in a preset time-sequence so that three ion-intensity ratios are measured in succession in a single GC/MS run. To increase the speed of analysis, this single measurement sequence is repeated on a second day. (Duplicate within- and between-day measurements are not performed as in the first IR/MS method.) A 0.5-percent agreement in the two results is required; if not achieved, a third measurement is made to obtain the agreement and the two results are used.

Task 1b. LITHIUM, MAGNESIUM, SODIUM, POTASSIUM, AND CHLORIDE

Summary: Although our last report covered the time period from April 1976 to October 1977, it was mentioned in the introduction that results obtained through September 1978 also had been included to "bring the information up to date". Thus, the information reported here may be somewhat duplicative in nature. The goals and selected data from the final statistical analyses are summarized in Task 1b, Table 1.

A. Lithium: Data information, collection, and analysis are complete and writing of the NBS-260 has begun.

B. Magnesium: Intra-laboratory exercises have been stopped until the magnesium gluconate dihydrate SRM is issued. A resolution of different interpretations of the statistical analyses of certification data has to be made before this work can continue. (See discussion in cover letter.)

C. Sodium: NBS Special Publication 260-60, "A Reference Method for the Determination of Sodium in Serum" has been issued.

D. Potassium: NBS Special Publication 260-63, "A Reference Method for the Determination of Potassium in Serum" has been completed (copy attached) and is being sent to the printers.

E. Chloride: The NBS special Publication 260 has been written and is undergoing internal review prior to publication.

Task 1b: Table 1. Imprecision and Bias Goals and the Values Obtained by Statistical Analysis from Interlaboratory Exercise Testing for Various Electrolytes.

Ion	Concentration (mmol/L)	LE	Imprecision[a] $\hat{\sigma}_{Total}$ (mmol/L)			Bias[b] $\bar{X}-X_{DM}$ (mmol/L)		
			Goal	Semiautomated	Manual	Goal	Semiautomated	Manual
Li	2.0	IV	0.1	0.017	0.038	0.2	-0.036	-0.030
Na	140.0	I	1.5	0.55	0.58	2.0	-0.4	-0.3
K	2.5	II	0.1	0.063	0.049	0.2	-0.027	0.025
Cl	98.5	II	0.3	0.063	0.049	0.2	0.042	0.065
CO2	100.0	II	1.0	1.01	0.52	2.0	-0.1	-0.2

[a] : precision = $\hat{\sigma}_{total} = \sqrt{\frac{\hat{\sigma}^2_{rep1}}{2} + \frac{\hat{\sigma}^2_{day}}{2} + \hat{\sigma}^2_{lab}}$

[b] Bias = $\bar{X} - X_{DM}$ where $\bar{X}$ is the average value for all labs and X_{DM} is the definitive method value.

Task Ic. LEAD (Pb) IN BLOOD

The graphite furnace procedure developed by T. C. Rains at NBS was compared to a chelation-extraction method used by Dr. E. Berman in routine clinical analysis. The primary purposes of this exercise, which took several months to complete, were (1) to investigate the long-term stability of the materials used in the intercomparison studies, (2) to compare the two methods when used on high Pb containing human blood samples, and (3) to provide within- and between-day data for statistical studies of repeatability.

Results of this exercise showed that Pb levels in the frozen porcine blood were stable over several months. Work with the human blood samples was started, but further samples and analyses are needed. The relative standard deviation for the within-day results ranged from 1.1% at the 75 µg/100 mL level to 13.5% at the 4 µg/100 mL level.

A round robin exercise to evaluate the written protocol and precision of the Rains method was initiated by contacting interested participants. Samples of five vials containing various levels of lead in blood were sent to 14 laboratories on May 15, 1978. The samples contained one vial of blood to be used during familiarization with the procedure plus four other vials containing blood with Pb levels ranging from about 4 to about 80 µg/100 mL.

Results from several labs were reported within three weeks. However, several labs did not follow the Rains' protocol

and several others did not respond to [illegible] letters. Labs were again contacted on [illegible], 1974, for a status report. Final results were requested.

While early reported values seemed to have sufficient precision, the variances between labs suggested the necessity of having isotope dilution-mass spectrometry (ID/MS) target values determined for the samples. This work was planned for late 19[illegible].

Task 1d. URIC ACID IN SERUM

The samples of six human pools to be utilized by one laboratory for definitive method as part of interlaboratory evaluation [illegible] put through the wet chemical processing steps and then analyzed measurements by GC/MS. Samples of the NBS reference serum have been similarly processed and also analyzed GC/MS. Data measurement was scheduled to begin in November 1980.

Task 1c. CONCLUSIONS

A. Comparison of II/M6 Results with the Empirical Equations

This subject was discussed in detail in [illegible] progress report although the work was done during the present report period.

Task 1f. SERUM IRON

A. Definitive Measurements of Total Iron

Iron in CDC Sera Lot Nos. 1777, 2277, and 2377 was determined by isotope dilution mass spectrometry. Three bottles of each lot were sampled for each determination and the combined aliquots were spiked with ^{57}Fe. The organic matrix was decomposed with perchloric and nitric acids (3:5) and the iron was separated by anion exchange chromatography. In an attempt to control the analytical blank variability, the collected fractions were further purified by ion exchange chromatography. The iron concentrations were then determined using thermal ionization mass spectrometry.

The data are shown in Task 1f, Table 1. The analytical blank contribution for this analysis was ±5 nanograms. This uncertainty component combined with those for the ratio determination (0.2%) and the spike calibration (0.1%) leads to an estimated accuracy of 0.4 percent for the analysis.

B. A Spectrophotometric Analysis of Iron in Frozen Serum Pools

Samples from seven different sites of serum were analyzed for iron using a slightly modified version of the procedure requested by I. L. Barnes (NBS number of Fe std./ 1000).

After allowing the frozen samples to come to room temperature, the samples were gently mixed and 3 mL aliquots were transferred to 15 mL ground glass centrifuge tubes. Precision

Task 1[a], Table 1. Iron in CDC Serum Samples

Lot No.	µg Fe/g	µmole Fe/L[b]
1777A	0.9370	17.77
1777B	0.9270	17.58
		Av. 17.68
2277A	0.5567	9.788
2277B	0.5562	9.779
		Av. 9.784
2377A	1.662	30.45
2377B	1.688	30.93
		Av. 30.69

[a]Calculated using the atomic weight of iron as 55.847 ± 0.3 the following serum densities:

Lot No.	Density
1777	1.0334
2277	1.0117
2577	1.0[illegible]

precipitating reagent[1] was added and, after mixing, the samples were heated to 56°C in a water bath for 15 minutes. After remixing, the samples were cooled to room temperature and centrifuged at ~1800 rpm's for 10 minutes. Aliquots of the supernatant were then transferred to other ground glass

[1]20 g trichloroacetic acid (G. Frederick Smith) + 6 mL thioglycolic acid (Sigma) + 12.2 mL HCl diluted to 200 mL with H_2O.

centrifuge tubes. Ferrozine chromogen solution[2] was added and after centrifuging again, the absorbance of the colored solution was read at 562 nm. Iron standards were prepared from SRM 917 according to directions obtained from J. J. Murphy (1968). Results are given in Task 1f, Table 2.

The only differences between the procedure suggested and the procedure actually used was that ground glass tubes were used throughout rather than the plastic tubes for the second step and an additional centrifugation step was added just before reading the absorbance. This was found to be necessary since any small particles of protein material which may get into the supernatant aliquot can cause spurious absorbance values if not centrifuged out.

Most of the results obtained in these analyses were lower than previous ones. We find that the isotope dilution analyses were indeed lower than the initial work done by this procedure, and these are lower still. We feel this may indicate that there is a problem with the samples, that they are gradually losing iron to the container walls or through some other step and that perhaps this should be seriously considered.

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[2] 27.2 g sodium acetate + 10 mg Ferrozine (Hach Chem. Co.) diluted to 100 ml with H_2O.

Task 15 Table [illegible]. Spectrophotometric [illegible]

Sample Number	Condition Description	Iron Values	
		[illegible]	[illegible]
1777	Normal Iron n = 10	$\bar{x}$ = .[illegible] σ = .[illegible]	16.90
2311	High Iron n = 6	$\bar{x}$ = 1.619 σ = .02[illegible]	31.81
2177	Low Iron n = 6	$\bar{x}$ = .591 σ = .006	19.6[illegible]
1877	Moderately Hemolyzed n = 6	$\bar{x}$ = 1.040 σ = .005	18.61
1977	Grossly Hemolyzed n = 6	$\bar{x}$ = 1.110 σ = .011	[illegible]
2177	High Bilirubin n = 6	$\bar{x}$ = .890 σ = .01[illegible]	[illegible]
2077	High Turbidity n = 6	$\bar{x}$ = .950 σ = .035	[illegible]

Task 1g. BILIRUBIN

No work was done on this analyte during this period.

Task 1h. UREA

In our preliminary work on an ID/MS method, we found that urea could be isolated from the serum samples and that the ratio of labeled to unlabeled molecules of urea in the isolated material could be measured after a direct probe insertion of the specimen into the mass spectrometer. The results obtained in the original experiment were within one percent of the expected values, and for the limited amount of time and effort put into the measurement we found reasonable precision (±1%) in the results; hence, no further study seemed to be needed prior to applying the method to samples requiring ID/MS analyses.

On attempting the measurements on inter-laboratory exercise pool samples and the WHO pool, we could not improve the precision beyond that attained previously, and we realized that the erratic results could be due to a lack of control over the temperature of the sample when it is in the probe of the mass spectrometer and hence over the evaporation of the urea sample. Provision was therefore undertaken to water-cool the probe for these measurements.

U.S. DEPT. OF COMM. BIBLIOGRAPHIC DATA SHEET	1. PUBLICATION OR REPORT NO. NBSIR 79-1707	3.

4. TITLE AND SUBTITLE

IDA Contract No. 74-58(0)
October 1977 to September 1979

5. Publication Date: August 1979

6. Performing Organization Code

7. AUTHOR(S)

I. Schaffer, R. A. Velapoldi and D. J. Reed

8. PERFORMING ORGANIZATION NAME AND ADDRESS

NATIONAL BUREAU OF STANDARDS
DEPARTMENT OF COMMERCE
WASHINGTON, DC 20234

11. Contract/Grant No.

9. SPONSORING ORGANIZATION NAME AND COMPLETE ADDRESS (Street, City, State, ZIP)

Bureau of Medical Devices and Diagnostic Products
Food and Drug Administration
Silver Spring, Maryland 20910

13. Type of Report & Period Covered

10. SUPPLEMENTARY NOTES

☐ Document contains a computer program; SF-185, FIPS Software Summary, is attached.

11. ABSTRACT (A 200-word or less factual summary of most significant information. If document includes a significant bibliography or literature survey, mention it here.)

This report describes work performed at the National Bureau of Standards from October 1977 through September 1979 in the continuation of projects initiated under IDA Contract 74-58(0). Some of the developments reported here were mentioned but not given in detail in the previous report in the series which was written after September 1978.

12. KEY WORDS (Six to twelve entries; alphabetical order; capitalize only the first letter of the first key word unless a proper name; separated by semicolons)

13. AVAILABILITY — Unlimited

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☐ Order From Sup. of Doc., U.S. Government Printing Office, Washington, D.C. 20402, SD Stock No. SN003-003-

☐ Order From National Technical Information Service (NTIS), Springfield, VA. 22161

14. NO. OF PRINTED PAGES

15. Price