MANUAL OF OPERATIONS

VOLUME 3, CHAPTER 5

CENTRAL AMINO ACID LAB MANUAL

Manual of Operations Volume 3, Chapter 5

Central Amino Acid Lab

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1.1 Central Amino Acid Laboratory and Clinical Center Responsibilities:

a. The Central Amino Acid Laboratory (CAAL) at the University of Iowa will implement and coordinate the plasma amino acid analyses of samples generated by the MDRD Clinical Centers. This includes supplying the Clinical Centers with mailing cartons, Centricon-10 ultrafiltration assemblies, tubes for shipping prepared plasma, tubes for holding plasma prior to transfer to tubes providing internal standards, tubes for storing extra plasma, tubes containing the internal standards (with and without sodium dodecylsulfate), and shipping forms for Airborne Express overnight package service.

b. The Clinical Centers will be responsible for the drawing of the blood samples, separation of plasma from erythrocytes, mixing plasma with internal standards in the presence and absence of sodium dodecyl-sulfate, deproteinization of the plasma using ultrafiltration and shipping the prepared samples on dry ice by Airborne Express to the CAAL.

1.2 Telephone and Written Communications:

a. Telephone inquiries having to do with aspects of amino acid analysis, quality control performance, amino acid analysis data forms, etc. may be directed to the CAAL at the University of Iowa (319-353-6481).

Calls to this number will generally be answered between 8 a.m. and 5 p.m. Central Time, weekdays only.

b. Written inquiries should be addressed to: CAAL, c/o Dr. Lewis D. Stegink, Department of Pediatrics, The University of Iowa, S-385 Hospital School, Iowa City, IA 52242. Alternatively questions can be sent by electronic mail.

c. The CAAL will communicate with participating Clinical Centers, if need be, at the addresses/phone numbers listed in the most recent MDRD Study Address Directory.

2.0 Evaluation of Sample Preparation at Clinical Centers:

At the beginning of the study, and at 6 month intervals during the study, the CAAL will send each Clinical Center a standard simulated plasma sample to prepare as part of the

quality control program. The Clinical Centers will be asked to prepare the samples according to the standard instructions and return the prepared samples to the CAAL for analysis. The analysis will help us spot potential errors in sample preparation at the Clinical Centers.

2.1 The overall scheme for preparation of the quality control sample is listed below and is summarized in Figure 1:

a. Step 1: Thaw the simulated plasma standard by placing the tube in room temperature water. Mix the thawed standard well by gently inverting the tube repeatedly, or by gentle use of a vortex mixer.

Using the Eppendorf or similar pipette, add exactly 1.00 ml of simulated plasma to each of 2 different test tubes containing internal standards (obtained from the CAAL). One tube will contain only the internal standards, the other tube will contain internal standards and sodium dodecylsulfate. The two test tubes are labeled, tightly capped, and then gently and repeatedly inverted for about 30 seconds.

b. Step 2: After being allowed to stand for 5 minutes at room temperature, with brief mixing at each minute interval, each of the two simulated plasma--internal standard mixtures (with and without sodium dodecylsulfate) is transferred to separate, labeled Centricon-10 Microconcentrator assemblies (provided by the CAAL). Centricon-10 assemblies are used one time only and then discarded.

c. Step 3: The remaining simulated plasma (extra plasma) is transferred to a purple "bullet tube" (supplied by the CAAL), labeled and frozen at -70° C until shipped to the CAAL.

d. Step 4: Both microconcentrator assemblies are then centrifuged at 3000 to 5000 x G until 300 to 400 µIL of protein-free ultrafiltrate are obtained (about 30 minutes).

e. Step 5: The ultrafiltrate from each microconcentrator assembly is tightly capped and the labeling checked. The samples are immediately frozen at -70° C. Samples may be held frozen at -20° C for a short period of time (12 hours) prior to storage of samples at -70° C.

f. Step 6: The frozen deproteinized samples of simulated plasma prepared by

ultrafiltration and the purple bullet tube containing the extra plasma are sent to the CAAL on dry ice at the following address: MDRD Central Amino Acid Laboratory,

c/o Dr. L. D. Stegink, Department of Pediatrics, S-385 Hospital School, The University of Iowa, Iowa City, IA 52242.

2.2 A discussion of the evaluation of these quality control samples is found in section 8.0 of this document.

3.0 Interaction of the Clinical Centers and the CAAL

a. Items Supplied by CAAL: The CAAL will supply each Clinical Center with mailing boxes and a supply of preaddressed labels for Airborne Express. Use of these labels allows the Clinical Centers to charge costs for shipping samples to the CAAL to the MDRD Central Amino Acid Laboratory account. The CAAL will also supply each Clinical Center with Centricon-10 membrane filter assemblies for deproteinization of the samples, tubes for storing the extra plasma, and special tubes containing internal standards with and without sodium dodecylsulfate. Note, the internal standard tubes must be kept frozen until used.

b. Need for Accuracy of Pipetting at Clinical Centers: It is critical that the pipetting of the heparinized plasma at the Clinical Centers be done with great care. The accuracy of all of the amino acid data depend upon this procedure.

c. Items Needed by Each Clinical Center: Each Clinical Center will need to supply heparinized blood tubes, disposable Pasteur pipettes with filling bulbs (or a similar pipette), an accurate 1.00 ml Eppendorf pipette (or similar device) with tips, a clinical centrifuge to separate plasma from erythrocytes, a centrifuge with a fixed angle head capable of developing 3000 x g (a swinging bucket head will not work), -70° C freezer space, and a dry ice supply. All other supplies will be provided by the Central Amino Acid Laboratory.

d. Storage of Prepared Samples and Shipping to the CAAL: Prepared plasma samples are to be stored at -70° C at each Clinical Center until shipped on dry ice by Airborne

Express to the Central Amino Acid Laboratory once a month or when the Clinical Center has accumulated 25 samples, which ever comes first. The only exception to this procedure is for samples from patients ingesting keto acids. These samples should be sent to the CAAL as soon as possible after having been drawn. However, if another keto acid sample will be drawn within a week of the first sample, the center may wait to send all the samples for the week in one shipment. The cost of the dry ice needed for shipping samples from the Clinical Centers to the Central Amino Acid Laboratory must be paid by each individual Clinical Center.

4.0 Sample Processing At The Clinical Centers

Drawing Blood Samples and Processing Plasma for Amino Acid Analysis

The overall scheme for sample preparation is shown in Figure 1. Note, label all tubes required to process a sample with patient name, number,etc. before beginning!

1. Blood samples (minimum of 5 ml) are drawn from subjects under appropriate conditions. Blood samples should be obtained after an overnight fast and before infusion of I^{125} -label used for GFR studies. Please note that fasting includes abstaining from ingestion to the ketoacid preparation. Blood samples must be drawn into heparinized tubes (to prevent clotting), the stopper firmly replaced and the tube inverted 4 or 5 times (to dissolve the heparin). If a heparin lock is used to draw the blood sample, remember to draw sufficient blood through the "lock" to remove the heparinized saline in the lock (and discard this blood--saline mixture) prior to drawing the blood sample to be prepared for amino acid analysis.
2. The whole blood is prepared immediately, (or if this is impossible it is held on ice for no longer than 15 minutes before centrifugation) by centrifuging at 2000 x G in a clinical centrifuge for 5 minutes to separate the erythrocytes (bottom layer) from plasma (upper layer). The plasma layer is carefully withdrawn and transferred to a labelled polystyrene tube using a Pasteur pipette (or other suitable pipette), taking special care to exclude the buffy coat layer (interface layer).

   If clotting is a problem in a particular laboratory and/or a particular patient, the plasma layer may be removed to another heparinized tube rather than to the

polystyrene tube provided. If this is done, make sure that the plasma is swirled gently to dissolve the heparin prior to withdrawing the two 1.00 ml samples from the pooled plasms for transfer to the tubes containing internal standards.

3. Using the Eppendorf or similar pipetting device, add exactly 1.00 ml of plasma to each of two labeled test tubes containing internal standards (obtained from the CAAL). One tube contains the internal standards with sodium dodecylsulfate (labeled SDS I.S.), the other tube contains only the internal standards (labeled I.S.). The tubes are capped, gently and repeatedly inverted for about 30 seconds, and then allowed to stand at room temperature for about 5 minutes.
4. The remaining plasma (extra plasma) is transferred to a purple bullet tube (supplied by the CAAL), labeled and frozen at -70° C until shipped to the CAAL.
5. Assemble and label two Centricon-10 Microconcentrator devices (provided by the CAAL). Transfer the contents of each of the two tubes containing plasma and internal standards (with and without sodium dodecylsulfate) to separate labeled Centricon-10 Microconcentrator devices (provided by the CAAL).

   The transfer of the contents of each of the two tubes containing plasma and internal standards to the ultrafiltration assemblies can be carried out using Pasteur pipettes; this transfer does not need to be quantitative.
6. Both microconcentrator assemblies are then centrifuged at room temperature in a fixed angle rotor at 3000-5000 x G until between 300 to 400 micro µIL of protein-free ultrafiltrate are obtained (about 30 min).
7. The top portion of each microconcentrator assembly is discarded. The filtrates obtained from the ultrafiltration of the plasma sample with and without sodium dodecylsulfate are transferred from the filtrate cups to the labeled, small, wide-mouthed, conical, plastic test tubes (supplied by the CAAL) and tightly capped.
8. The capped, labeled tubes are firmly tied together with a rubber band and then attached to the corresponding labeled purple bullet tube containing the extra plasma and stored at -70° C until the 3 samples are sent as one unit on dry ice by Airborne Express the CAAL at the following address: MDRD Central Amino Acid Laboratory,

c/o Dr. L. D. Stegink, Department of Pediatrics, S-385 Hospital School, The University of Iowa, Iowa City, IA 52242. Be sure to include a completed MDRD Form 19 for each sample in the shipping container.

5.0 Sample Processing at the CAAL

5.1 Sample Receipt and Log-In

a. CAAL personnel will notify Dr. Stegink or Mr. Brummel when MDRD samples arrive.

b. CAAL personnel will log-in the samples in the bound notebook of the CAAL. Information recorded includes: Master ID number [assigned as entry is made and numbered consecutively before the last two digits of the current year (i.e. 234-89)], patient name, patient number, initials of individual logging the sample in, sample type (plasma, urine, CSF), sample condition, physicians name and/or study number, date sample received and the date that the analyses (usual amino acids, alloisoeucine, free tryptophan and total tryptophan) are completed.

c. During the log-in process CAAL personnel will check MDRD Form 19 and all sample tubes closely for any errors, discrepancies or other problems. These will be recorded in the log.

d. CAAL personnel will notify the Clinical Centers by electronic mail of any problems.

e. MDRD Form 19 for each sample will be filed in the MDRD Study file at the CAAL.

5.2 Sample Storage at the CAAL

a. Prepared plasma samples for analysis of free amino acids, total tryptophan and the extra plasma samples will be labeled and stored at -70° C until analyzed.

b. When ready to be analyzed, prepared plasma samples will be thawed, mixed well and analyzed using an amino acid analyzer and a high performance liquid chromatograph (HPLC).

5.3 Sample Preparation and Handling at CAAL

a. When received, samples will be logged as described above and stored frozen at -70° C until assay. At that time samples will be thawed, mixed well and treated as shown in

Table 1.

b. A 50 µlL sample of the plasma ultrafiltrate treated with sodium dodecylsulfate will be injected on the Beckman 6300 amino acid analyzer using the normal buffer system to determine levels of the usual amino acids. The concentration of each free amino acid will be appropriately adjusted for the recovery of -aminoethylcysteine which is included in the internal standard test tube used in sample preparation at the Clinical Center.

c. An aliquot (20 µL) of the plasma ultrafiltrate not treated with sodium dodecylsulfate will be injected on the HPLC for analysis of the free tryptophan content. A second 20 µIL aliquot of the plasma ultrafiltrate treated with sodium dodecylsulfate will be injected on the HPLC for analysis of total tryptophan concentration. Protein-bound tryptophan will be determined by the difference between free and total plasma tryptophan values. The amount of tryptophan found by HPLC will be appropriately adjusted for the recovery of α-methyl-DL-tryptophan which is included in the internal standard test tube used in sample preparation at the Clinical Center.

5.4 Instruments and Methodology Used for Amino Acid Analysis

a. The CAAL uses a Beckman System 6300 high performance amino acid analyzer with a physiological fluid analysis program requiring 126 minutes per analysis. Data are acquired by use of a Hewlett Packard 3390A integrator; each chromatogram is visually inspected as the data are recorded. In this chromatographic system, alloisoleucine and cystathionine coelute. This normally is not a problem since only trace quantities of these amino acids are usually present in plasma. However, plasma alloisoleucine levels are elevated in maple syrup urine disease (MSUD) and plasma cystathionine levels are elevated in both premature infants and in children with cystathioninuria. Thus, when the size of the peak at the cystathionine--alloisoleucine position is larger than normal, a second aliquot of deproteinized plasma will be analyzed on the Beckman 6300 analyzer using a special analysis program that separates these two amino acids (at the expense of the resolution of several other amino acids). This method separates alloisoleucine from other amino acids present in plasma.

b. Total tryptophan can be quantitated using standard amino acid analyzer methodology

when plasma is treated with sodium dodecylsulfate (SDS) prior to deproteinization. However, total and free tryptophan are best determined by chromatography of specially prepared aliquots of plasma using an HPLC method; protein-bound tryptophan is determined by difference. In order to analyze for both free and total tryptophan, two aliquots of each plasma sample are deproteinized by ultrafiltrations using Centricon-10 (Amicon Corp.) centrifugal filter assemblies. In one case the plasma is not pretreated in any way before ultrafiltration, in the other the plasma is treated with sodium dodecylsulfate (releases protein-bound tryptophan) prior to ultrafiltration.

c. Description of tryptophan method: We use an HPLC assay to determine plasma tryptophan. Our method is a modification of a previously published method. We use a reversed phase column (4.6 x 250 mm, 5) with the following mobile phases. Buffer A is prepared by mixing 200 ml of buffer B plus 100 ml methanol. Buffer B is prepared by mixing 550 ml HPLC grade water, 50 ml HPLC grade methanol and 5 ml of lithium acetate buffer (4.0 N; pH 4.7). The program starts with 100% B buffer as the mobile phase for one minute with a linear ramp to 60% B--40% A from 1 minute to 16 minutes. The mobile phase flow rate is 2.0 ml/minute. The UV detector is set at 280 nm. Data are acquired by use of a Hewlett Packard 3390A integrator; each chromatogram is also visually inspected as the data are recorded.

5.5 Reporting of the Data

a. The computer analysis of the amino acid analyzer chromatogram will be recorded on the standard CAAL amino acid analysis worksheet. The chromatogram will be visually inspected to assure proper function of the computer for integration of the peaks. The data will then be transferred to MDRD Form 36. The data transfer will be checked for accuracy by CAAL personnel.

b. The analytic data will be provided to the Data Coordinating Center (DCC) of the MDRD Study (using the Datalex program and modem) within three weeks of receipt of the sample, assuming that the samples are submitted uniformly across the 4 year period of this study.

c. The portion of each sample remaining after analysis will be kept frozen at -70° C for

one year after sample receipt.

d. Quarterly reports will be prepared showing quality control results and results of analyses of samples, including problems encountered in receipt and analysis of samples.

Changes in analytic technique will be documented, including data on their effect on precision of the data.

6.0 Quality Control

6.1 Internal Quality Control: Beckman 6300 Amino Acid Analyzer and HPLC Chromatograph

a. A calibration mixture is formulated from solutions of standard amino acids supplied by Beckman Instruments (System 6300 STD, AN^+, and B^+) supplemented with asparagine, glutamine and tryptophan, to yield a solution containing the amino acids shown in Table 2 at the concentrations listed. In addition the unnatural amino acids S-aminoethylcysteine and α-methyl-DL-tryptophan are added as internal standards.

b. A second, separate calibration mixture is formulated from solutions of standard amino acids provided by Beckman Instruments Co as listed above, but is supplemented with alloisoleucine for calibrating the Beckman 6300 amino acid analyzer when the special program separating alloisoleucine from cystathionine is used.

c. Aliquots of each of these standard solutions are placed in small tubes under nitrogen and stored frozen at -70° C until use. At that time the Beckman 6300 amino acid analyzer is calibrated with the appropriate standard before a given series of plasma samples is analyzed. The integrator is placed in the internal standard mode and all subsequent calculations are based on the recovery of the internal standard, S-aminoethylcysteine, which elutes between the hydroxylysines and ornithine. In this way any variation in color yield resulting from ninhydrin degradation or other variables can be corrected, assuming that all amino acids behave similarly to the internal standard.

d. Alpha-methyl-DL-tryptophan does not react well with ninhydrin and as a result does not give a significant interfering peak in the elution area between tryptophan and ammonia

where α-methyltryptophan elutes when using standard Beckman 6300 amino acid analyzer methodology.

e. The procedure for "on site" sample preparation at the clinical Centers calls for the use of a test tube containing internal standards (with and without sodium dodecylsulfate) to which 1.00 ml of plasma must be added. The internal standard tubes contain an amount of S-2-aminoethylcysteine and α-methyltryptophan which should yield a value of 10.0 μmole/dL upon assay when dissolved in 1.00 ml plasma. The internal standard tubes providing sodium dodecylsulfate contain 2g of sodium dodecylsulfate in addition to the internal standards. All Beckman 6300 chromatograms are corrected for recovery of S-aminoethyl-cysteine; HPLC chromatograms are appropriately corrected for recovery of α-methyltryptophan.

f. The between sample variability of the amino acid analyses, as a result of day-to-day changes in the ninhydrin reagent, will be assessed by the inclusion of a standard sample of deproteinized plasma with each batch of patient samples analyzed on the Beckman 6300 amino acid analyzer. This standard sample is an aliquot of a large volume of deproteinized plasma stored at -70° # C in small aliquots. Runs of samples in which the amino acid totals of this standard sample differ from the mean totals for this standard by more than + 10% will be analyzed until the standard sample values are within this range.

g. HPLC analysis for tryptophan involves preparation of a standard curve prior to analysis of each set of plasma samples. Acquisition of the data uses a Hewlett Packard 3390A integrator. Our data indicate there is little if any 280 nm absorbing compound eluting from the HPLC column at the position corresponding to α-methyl-tryptophan when plasma samples are assayed as described. Tryptophan has been shown to bind to serum albumin. The binding requires tryptophan's carboxyl group, certain areas of the indole ring and a sterically bulkless α-carbon atom. Since α-methyltryptophan is most like tryptophan chemically and since it does not release bound tryptophan from albumin, we have chosen it as an internal standard for assays of tryptophan. It is completely resolved from tryptophan by the HPLC method used, does not suffer from undue photosensitivity as do some indoles, and does not appear to have erratic recoveries in

the presence of plasma treated with sodium dodecylsulfate.

6.4 Formulation of Internal Standard Solution:

S-2-Aminoethyl-L-cysteine monohydrochloride (#A2636) and α-methyl-DL-tryptophan (#M8377) are purchased from Sigma Chemical Co and are used to make a stock solution (0.100 M of each in 0.1 N NaOH) which is saturated with nitrogen. Exact 0.500 ml aliquots of this solution are put into small polystyrene test tubes and capped after the air over the liquid is replaced with nitrogen. These aliquots are stored frozen at -70° C in the dark until they are reconstituted for use. At that time one aliquot is quantitatively diluted to 50.0 ml with HPLC-grade water.

a. Test tubes containing internal standards sent to the Clinical Centers are prepared by lyophilizing exactly 0.100 ml of the reconstituted solution onto the bottom of 12 x 55 mm polystyrene tubes. After lyophilization 10 µL of a 20% solution of sodium dodecylsulfate is placed on the side wall of the test tube and allowed to air dry. The air in each tube is carefully replaced by nitrogen, the tubes are capped with a polyethylene stopper and stored at -20° C degrees until use. Random units are selected throughout a batch and assayed for content. In addition, units are checked monthly to assure integrity of content.

7.0 External Quality Control:

Twice a year, the local on site Clinical Center Laboratory technicians will draw an additional blood sample from a patient, prepare it, label each sample, and send both specimens to the Central Amino Acid Analysis Laboratory for analysis. Duplicate results will be compared.

8.0 Quality Control of Clinical Center Sample Preparation:

It is critical that sample preparation at the Clinical Centers be accurate. To check this point, we will submit a simulated plasma sample of known composition and containing 10 µmoles/dL added alloisoleucine as internal standard to each Clinical Center both prior to the start of the study and every 6 months thereafter. The Clinical Centers will prepare the simulated plasma and return the prepared solutions to the Central Amino Acid Laboratory.

Systematic errors in sample preparation will be detected by calculation of S-aminoethylcysteine and α-methyltryptophan concentration usage alloisoleucine concentration as the internal standard.

8.1 Review of Data Evaluating Clinical Center Pipetting Methods

a. After performing an amino acid analysis on the prepared simulated standard plasma samples from each Clinical Center, Dr. Stegink and Mr. Brummel will review the results and compare the amino acid concentrations observed to actual concentrations present in the standard.

b. Results of analyses in which amino acid concentrations are within acceptable limits will be transmitted to the DCC using Datalex Form 36.

c. For results outside of the acceptable limits the following action will be taken:

   1. The Clinical Center will be informed that the results fell outside acceptable limits.
   2. The CAAL will mail out another quality control sample for preparation and return to the laboratory.

d. After the second sample has been prepared, returned to the CAAL and analyzed, the results will again be reviewed by Dr. Stegink and Mr. Brummel. If the problem has been corrected and the results now fall within acceptable limits, no further action is necessary. If the second set of results also fall outside of the acceptable limits the following actions will take place:

   1. The Clinical Center involved will be informed that the second set of values are outside of acceptable limits.
   2. Dr. Stegink will inform the Chairman of the Quality Control Committee in writing of the problem. The Chairman will review the problem with the Committee.
   3. The Quality Control Committee will work with the Clinical Center Principal Investigator to resolve the problem.

8.2 Duplicate Samples From Clinical Centers

a. Every 6 months, each Clinical Center will send one duplicate sample to the CAAL. The CAAL will analyze the samples and compare the results.

b. The data will be entered on Datalex form 36 and transmitted to the DCC.

c. Review of results:

1. The CAAL will prepare a report comparing the duplicate quality control results.
2. The report will be sent to the Quality Control Committee.
3. Results which vary by an absolute difference of more than 10% CV will be reviewed by the principal investigator of the CAAL and repeat analyses will be carried out if sample is available.

9.0 Review of Unusual Plasma Amino Acid Values

9.1 Sample Mishandling

a. After amino acid analyses of plasma samples sent by the Clinical Centers have been completed, Dr. Stegink and Mr. Brummel will review the values to look for evidence of sample mishandling. This evaluation will focus on a selected group of amino acids where sample mishandling markedly and obviously affects plasma concentrations. For example, plasma taurine concentrations will be elevated if a part of the buffy coat layer was included in the plasma sample (due to the high taurine content of the platelets). Similarly, plasma glutamate concentrations will be elevated and plasma glutamine values decrease when a blood sample is allowed to stand at room temperature prior to separation of the plasma from the erythrocytes; under these conditions plasma cystine concentrations are also decreased. Similar effects on glutamate, glutamine and cystine concentrations are observed if the plasma sample has been allowed to stand for an excessive amount of time prior to ultrafiltration. Finally, hemolysis of the blood releases the enzyme arginase from the erythrocyte (arginase is not usually found in plasma). The arginase catalyzes the conversion of plasma arginine to ornithine, increasing plasma ornithine concentrations and decreasing plasma arginine concentrations.

b. If unusual concentrations of these amino acids are noted in a given plasma sample, amino acid analysis will be carried out on the portion of the corresponding plasma sample not treated with sodium dodecylsulfate to see if unusual values are still noted.

c. The Clinical Center submitting the sample will be contacted to see if conditions during

sample processing of that particular sample might have produced the unusual concentrations of the amino acids in question.

d. If an appropriate reason for the unusual value(s) can be ascertained, the values from this sample will be excluded from the data base and the Clinical Center involved will be asked to use greater care in preparing the blood samples. If an appropriate reason cannot be ascertained, the values from the sample will be included in the database and sent to the DCC via Datalex since there are no statistical reasons for excluding the data.

e. If the problem of unusual values occurs in only a few samples from a given Clinical Center, the Clinical Center will be notified and asked to use greater care in preparing the samples. If values from a given Clinical Center continue to show unusual values not seen in samples from patients at other Clinical Centers on that same diet, the following actions will take place.

   1. The Clinical Center involved will be informed of the problem.
   2. Dr. Stegink will inform the Chairman of the Quality Control Committee in writing of the problem. The Chairman will review the problem with the Committee.
   3. The Quality Control Committee will work with the Clinical Center Principal Investigator and CAAL personnel to resolve the problem.

9.2 Unusual Correction Values For Recovery Of Internal Standards

a. The actual chromatographic values obtained by Beckman HPLC and 6300 amino acid analyzer methodologies are corrected for recovery of the internal standards mixed with the plasma at the Clinical Centers prior to ultrafilatration. If the correction observed upon analysis varies more than 20% from the correction normally observed by the CAAL, the sample will be reanalyzed. If the second analysis still shows a correction of more than 20% from the correction normally observed by the CAAL, the corresponding plasma sample not treated with sodium dodecylsulfate will be analyzed by Beckman 6300 methodology. If the correction for internal standards is within the 20% range, the new values will be included in the data set. If the correction for internal standard is still greater than 20% of the correction normally observed by the CAAL, the values will be excluded from the data set.

The Clinical Center providing the sample will be appraised of the problem and CAAL personnel and the Clinical Center staff will attempt to find the cause of the problem.

b. If the problem only occurs in a few samples from a given Clinical Center, the Clinical Center will be notified and a review of the sample preparation method will be carried out. If the values for a given Clinical Center continue to show unusually high correction values not seen in samples from other Clinical Centers (relative to CAAL values), the following actions will take place.

   1. The Clinical Center involved will be informed of the problem.
   2. Dr. Stegink will inform the Chairman of the Quality Control Committee in writing of the problem. The Chairman will review the problem with the Committee.
   3. The Quality Control Committee will work with the Clinical Center Principal Investigator and the CAAL personnel to resolve the problem.

6300 Amino Acid Analyzer

Calibrate with ususal standard mixture of amino acids plus tryptophan, glutamine and asparagine.

↓

Analyze plasma ultrafiltrate treated with SDS using standard analysis program to obtain levels of most amino acids

when required

↓

Calibrate with usual standard mixture of amino acids plus added alloisoleucine

↓

Analyze plasma ultrafiltrate treated with SDS using special analysis program to separate alloisoleucine from cystathionine

HPLC System

Calibrate with tryptophan standard

↓

Analyze plasma ultrafiltrate not treated with SDS for values of free tryptophan

↓

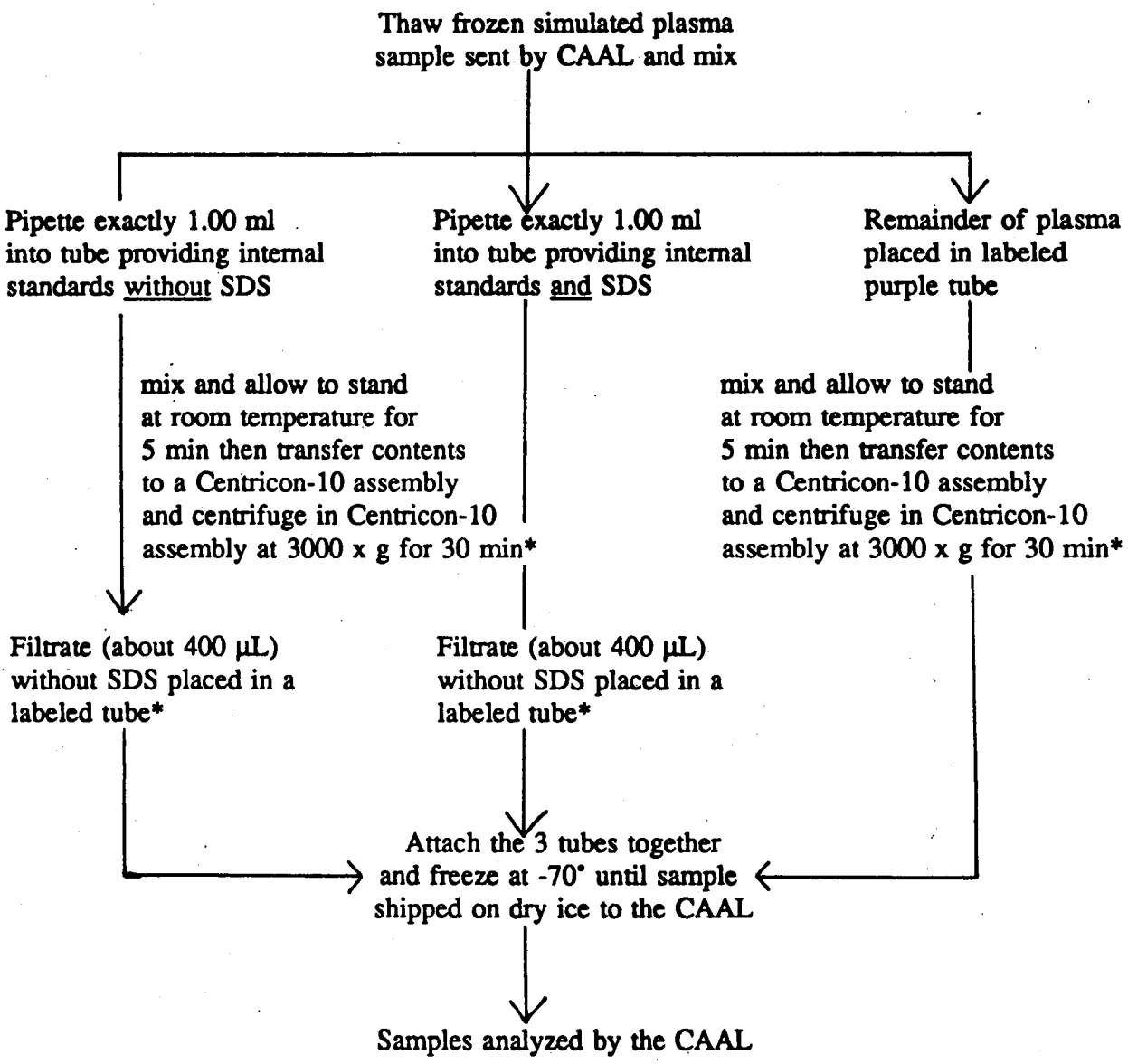
Analyze plasma ultrafiltrate treated with SDS for values of free tryptophan

Table 2: Concentrations of amino acids present in the standard amino acid calibration mixture used.

Phosphoserine 50	
Taurine	50
Phosphoethanolamine	50
Urea	1550
Aspartic acid	100
Hydroxyproline	100
Threonine	100
Serine	100
Glutamic acid	100
Glutamine	100
Sarcosine	100
Alpha-aminoadipic acid	25
Proline	100
Glycine	100
Alanine	100
Citrulline	25
Alpha-aminobutyric acid	25
Valine	100
Cystine	100
Methionine	100
Cystathionine (Alloisoleucine**)	50
Isoleucine	100
Leucine	100
Tyrosine	100
Phenylalanine	100
Beta-alanine	100
Beta-aminoisobutyric acid	100
Homocystine	100
Gamma-aminobutyric acid	100
Ethanolamine	100
Tryptophan	100
Alpha-Methyltryptophan (int std)	100
Ammonia	100
Hydroxylysine	100
Allo-hydroxylysine	100
S-aminoethylcysteine (Int Std)	125
Ornithine	100
Lysine	100
1-Methylhistidine	100
Histidine	100
3-Methylhistidine	100
Anserine	100
Carnosine	100
Arginine	100

**When required

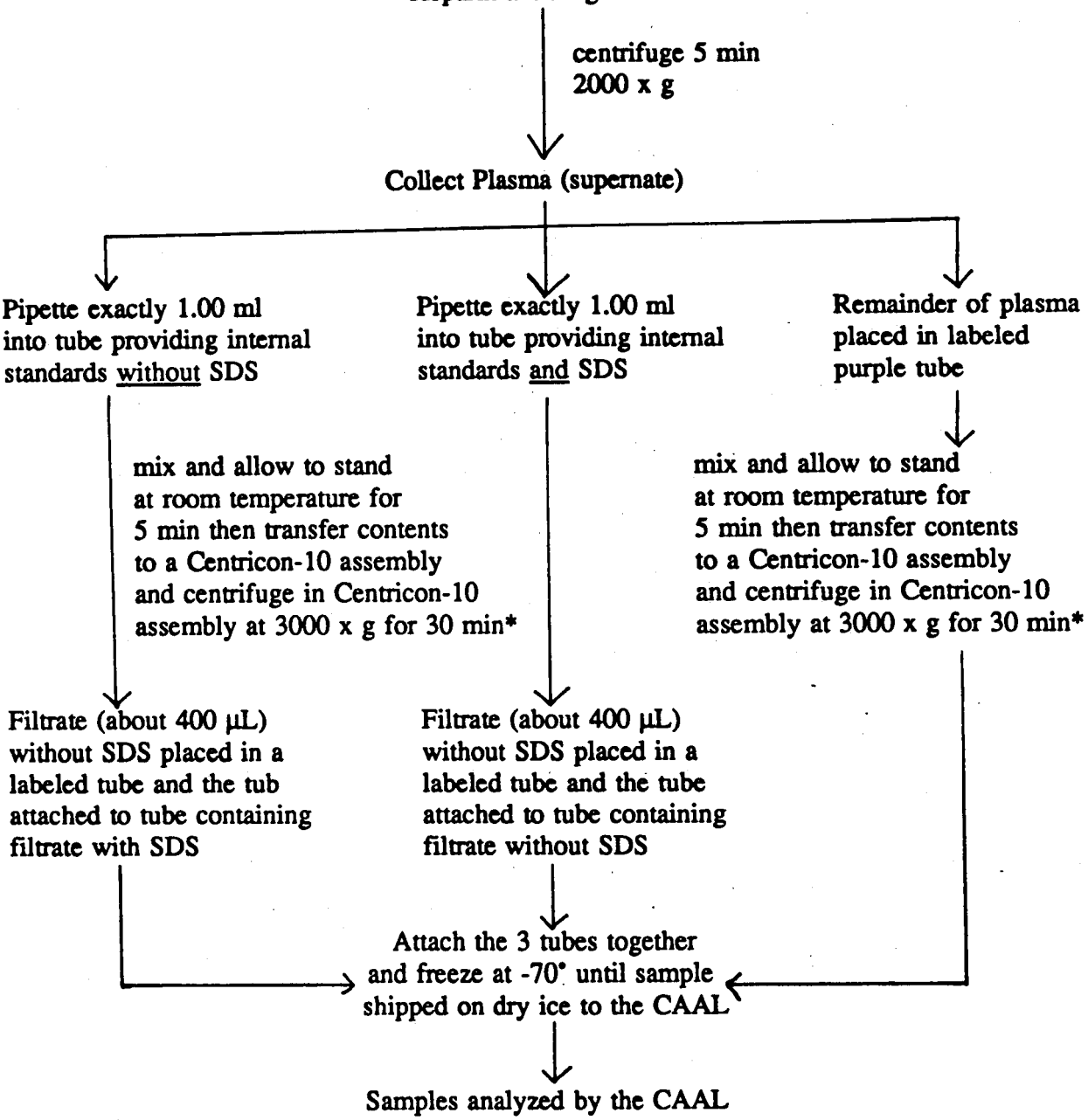
Figure 1: Scheme for handling quality control sample at the Clinical Centers



Abbreviations: SDS, sodium dodecylsulfate; CAAL, Central Amino Acid Laboratory.

*Note: Centricon-10 ultrafilters are discarded after use.

Figure 2: Scheme for sample handling at the Clinical Centers



Abbreviations: SDS, sodium dodecylsulfate; CAAL, Central Amino Acid Laboratory.

*Note: Centricon-10 ultrafilters are discarded after use.