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Note

Amino acid analysis of plasma: studies in sample preparation

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Deproteinization has been consistently recognized as a prerequisite step for amino acid analysis of plasma samples. A variety of chemical methods have been used to deproteinize biological fluids prior to amino acid analysis. Stein and Moore [1] adapted the Hamilton and Van Slyke picric acid method [2], which required a high level of laboratory technique and time. Hamilton et al. [3,4] endeavored to simplify plasma sample preparation for amino acid analysis by using 5-sulfosalicylic acid (SSA), but they pointed out that SSA is not as complete a protein-precipitating agent as picric acid. However, the remaining protein in the sample after SSA treatment did not impair analysis of free amino acids on 0.9-cm bore columns, and there appeared to be very little protein build up on the column until several hundred assays had been performed. Modern amino acid analyzers use 0.3-cm bore columns which are much more susceptible to protein interference than the larger diameter columns [5]. Other chemical methods of deproteinization of plasma samples prior to amino acid analysis are reviewed by Ohara and Ariyoshi [6]. They included the use of trichloroacetic acid, acetic acid, tungstic acid, ethanol, acetone, uranyl acetic acid, zinc/sulfuric acid and barium hydroxide. They indicated that picric acid, SSA and ethanol are acceptable for removal of protein before measuring free amino acids in plasma or serum by ion-exchange chromatography. However, the recovery of free amino acids after ethanol treatment is quite variable. Also, we have noted considerable plasma protein retention after precipitation with ethanol and related organic solvents [7]. This paper pre-

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sents a combined deproteinization of EDTA plasma with SSA followed by centrifugal ultrafiltration.

EXPERIMENTAL

Materials

Amino acid standards: Beckman System 6300 calibration standards B and An+ (Beckman Instruments, Palo Alto, CA, U.S.A.) and L-glutamine, DL-asparagine, DL-tryptophan, D-glucosaminic acid (Sigma, St. Louis, MO, U.S.A.).

Vacutainer tubes: EDTA (K_3 5 ml No. 6536, EDTA (K_3) 2 ml No. 6384 (Becton Dickinson, Rutherford, NJ, U.S.A.).

SSA (3.5 g per 100 ml) and Lowry [8] protein assay kit (Sigma).

Centrifuge filtration system: Centrifree System (30 000 MWCO) [9] from Amicon (W.R. Grace, Danvers, MA, U.S.A.).

Polypropylene sample cups (2 ml) for pH adjustment and centrifuge tubes: Sarstedt (Princeton, NJ. U.S.A.).

Amino acid analyzer: System 7300, with 25-cm column, Li-A, Li-D, Li-E and Li-F lithium elution buffers, Li-S sample dilution buffer, 1.0 a.u.f.s., 15.2 cm/h integrator real time plotter chart rate, 50-μl sample injection loop (Beckman Instruments).

Sample preparation

Blood is drawn from the antecubital vein in a 2- or 5-ml EDTA Vacutainer tube. Plasma is separated from cells by centrifugation at 2000 *g* for 15 min at 3 °C.

Fresh EDTA plasma (200 μl) is placed in a 0.6-ml centrifuge tube to which we add 20 μl of SSA [35% (w/v)] to give 3.5 mg of SSA per 100 μl of plasma. The tube is capped, vortexed for 5 s, allowed to stand for 5 min at room temperature, then centrifuged at 11 200 *g* for 5 min. The supernatant is removed and processed for analysis or is frozen at −20 °C for later processing. Preparation to this point must be made within 1 h from the time blood is drawn.

The supernatant is placed in the Centrifree microfilter reservoir, capped and centrifuged for 30 min at 1800 *g*. A 200-μl aliquot is then mixed with 200 μl of D-glucosaminic acid [10] (0.1 mg in 2.00 ml of pH 2.2 Beckman Li-S buffer) as the quality control standard (not an internal standard) to provide quantitative proof of the amount of sample injected. A volume of 150–300 μl of this sample is drawn into the loading loop of the Beckman System 7300 amino acid analyzer for the 50-μl measuring loop injection. Amino acid concentrations are determined by external standardization from the Beckman amino acid standards plus added asparagine, glutamine and tryptophan.

Amino acid analyzer operating parameters

The Beckman operating program No. 121 for physiological fluids with the system 7300 amino acid analyzer uses a sequence of three temperatures, four buffers (Li-A, Li-D, Li-E and Li-F) and a 25-cm column. The modifications of this operating program used in this study are listed in Table I. B5 is bypassed and B6 is the 0.3 *M* lithium hydroxide regeneration step. Standardization of the System

TABLE I

AMINO ACID ANALYZER OPERATING PARAMETERS

Parameter		Time (min)	Start-up (min)	Column temperature (°C)
Solvent to detector (S)	S	185	−18 Count	
Temperature (T)	T1	186	−17 down	38.0
Regeneration (B6)	B6	186	−17 steps	
Buffer change (B)	B1	188	−15 prior	
Ninhydrin to detector (N)	N	194	−9 to	
Recycle program (R)	R	202	−1 first	
Inject sample (I)	I	0	0 assay	
	T2	20.5		66.5
	B2	47.2		
	B3	112		
	B4	118		
	T3	143		70.0

Changes in the Model 7000 files:
I Files 1-4: end 196 min
A Files 1-4: stop 186 min
M File 1: end 186 min
A File 3: minimum width 0.2
(Conditions for run No. 1081)

7300 unit is by external standardization. Ninhydrin is made up as stated from the Beckman 7300 operating manual, but stored at 3–5 °C for 16 h prior to use, to avoid the super sensitivity period of freshly made up ninhydrin.

The remaining traces of protein after precipitation and filtration of the plasma samples (5–10 μg per injected sample) that go onto the analytical column are removed after 150–200 assay runs by reversing the analytical column and while directing the column effluent into a beaker, pumping a 0.3 *M* lithium hydroxide wash at 70 °C for 2.5 h through the column. This is followed by a 30-min equilibration period with Li-A buffer at 70 °C. The bottom of the column is then reconnected to the detection system. The need for this treatment is indicated by either back-pressures of 4.14 MPa or more over the usual 15.2 MPa, or by a loss of resolution in the threonine–serine–asparagine area of the chromatogram.

RESULTS AND DISCUSSION

Table II shows the protein concentration remaining in the supernatant after precipitating pooled EDTA-treated plasma samples at four different ranges of protein concentration, and precipitated with 3.0–10.0 mg SSA per 100 μl of plasma. These results indicate that there is no advantage of using more than 3.5 mg of SSA per 100 μl of plasma. This conclusion is confirmed with data from fresh individual plasma samples ($n=16$), which gave a mean $\pm$ S.D. of 326 ± 118 μg/ml with SSA at 3.5 mg per 100 μl of plasma and 270 ± 110 μg/ml with SSA at 4.0

TABLE II

PROTEIN CONCENTRATION REMAINING IN PLASMA SUPERNATANT AFTER PRECIPITATION WITH VARYING SULFOSALICYLIC ACID CONCENTRATION IN POOLED PLASMA SAMPLES WITH DIFFERENT INITIAL PROTEIN LEVELS

Initial level of plasma protein (g/dl)	Protein remaining (μg/ml)					
	3.0	3.5	4.0	4.5	5.0	10.0*
5.2–5.6	438	398	379	286	274	212
5.9–6.0	473	244	224	199	235	249
6.6–6.8	494	389	326	319	257	240
7.3–8.6	2463	229	239	217	157	162
Mean	967	315	292	255	231	216

*Amounts of SSA per 100 μl of plasma (mg).

mg. Utilizing SSA at 3.5 mg per 100 μl of plasma, but then buffering to pH 2.2 before precipitation of serum proteins as proposed by Mondino et al. [11], the residual supernatant protein concentration was 3142 ± 42 μg/ml on five individual fresh plasma samples. This illustrates that a pH adjustment of the SSA to obtain the desired sample pH, prior to protein precipitation, considerably diminishes the protein precipitating capability of SSA. Supernatants obtained with fresh plasma samples (less than 1 h old) prepared with 3.5 mg of SSA per 100 μl of plasma have pH values of 1.8–1.9. This value can be easily raised to pH 2.0–2.2 for the desired sample pH for addition to the ion-exchange column by dilution with Li-S buffer. EDTA plasma samples that are allowed to stand for 2 h or more have a pH of 1.3–1.4 after SSA precipitation. The results of Perry and Hansen [12,], Sahai and Uhlhaas [13] and Schaefer et al. [14] clearly indicate that the plasma samples should be processed through the SSA preparation step as soon as possible after venipuncture. Filtration through Amicon centrifuge tubes after SSA treatment removes approximately 80–100 μg/ml more of the residual supernatant protein. This filtration step allows double the number of assay runs before the column reversal and regeneration procedure must be applied.

This centrifugal filtration [9] process on untreated plasma or serum gives very comparable amino acid analysis results, to the combined SSA and centrifugal filtration process, but filtration by itself allows only a 50% recovery volume of sample supernatant. This added sample volume recovery becomes very critical with pediatric or small animal specimens.

Table III compares the variations (%) of the amino acid values from plasma samples prepared for analysis with and without Centrifree filtration after the SSA precipitation step. The greater variation in glutamine and tryptophan occurs during the SSA precipitation and is not increased by the filtration step.

Fig. 1 shows the chromatograms from the same column that show the high resolution obtained on two very similar human plasma samples from runs 9 and 1081, i.e. 1072 assay runs later.

The use of D-glucosaminic acid as a quality control standard, when a value of

TABLE III

MEAN PERCENTAGES DIFFERENCE OF FREE AMINO ACID VALUES FOR 35% SSA PREPARED PLASMA WITH/WITHOUT AMICON CENTRIFREE FILTRATION ($n=5$)

Amino acid	Difference (%)	Amino acid	Difference (%)
Phosphoserine (PSER)	3.6	Glycine (GLY)	2.3
Taurine (TAU)	2.8	Isoleucine (ILEU)	3.1
Phosphoethanolamine (PETN)	6.0	Leucine (LEU)	2.4
Urea	1.4	Tyrosine (TYR)	2.6
Hydroxyproline (HYP)	2.4	Phenylalanine (PHE)	7.5
Threonine (THR)	2.9	β-Aminoisobutyric acid (BABA)	9.5
Serine (SER)	2.4	Tryptophan (TRY)	13.6
Asparagine (ASN)	2.6	Ornithine (ORN)	1.6
Glutamic acid (GLU)	3.0	Lysine (LYS)	1.4
Glutamine (GLN)	11.2	1-Methylhistidine (1-MHIS)	8.8
Sarcosine (SAR)	8.5	Histidine (HIS)	5.0
α-Aminoadipic acid (AAD)	4.3	Arginine (ARG)	2.2
Proline (PRO)	1.6		

25 ng/ml has been set, has given us values between 24 and 29 over some 100 runs. The reasons for these elevated values are described later.

The amount of protein remaining in pooled EDTA plasma samples (Table II) after precipitation with 3.5 mg SSA per 100 μl of plasma is similar to the amount of protein remaining after use of 10.0 mg SSA per 100 μl of plasma, the greatest difference being 186 μg/ml. However, the lower 3.0 mg SSA per 100 μl of plasma is incapable of precipitating the high plasma protein concentrations in the 7.3–8.6 g/dl range. Protein precipiation with 4.0 or 3.5 mg SSA per 100 μl of plasma results in a mean difference of only 56 μg/ml. The 3.5 mg SSA per 100 μl concentration provides a pH value of 1.8–1.9 ($n=16$) with fresh EDTA plasma which is closer to the desired pH of 2.0–2.2 than the lower pH of 1.6–1.7 using 4.0 mg SSA per 100 μl of plasma. Heparinized plasma, less than 1 h old, after SSA treatment gave supernatants with pH values of 1.6–1.8 which complicates pH adjustment with Li-S buffer to the desired sample pH of 2.0–2.2. Also, the work of Konstantinides et al. [15] gave higher values for 10 out of 23 amino acids in the same samples collected in EDTA versus heparin.

SSA has replaced cumbersome chemical methods for protein precipitation from blood samples, either to study the protein precipitated [16] or the amino acids left in the supernatant [17]. SSA by itself does not provide a protein-free filtrate. We propose the use of the lowest amount of SSA for effective protein precipitation (3.5 mg per 100 μl of plasma) together with centrifugation through a microfilter to remove additional protein from the SSA supernatant without altering the recovery of free amino acids. The shifting peak positions for aspartic acid, threonine and serine, due to pH values that are too low, as noted in the past [5] using the customary amounts of SSA (five to ten times the amount used in this study), are essentially solved by decreasing the amount of SSA to 3.5 mg per 100 μl of fresh plasma.

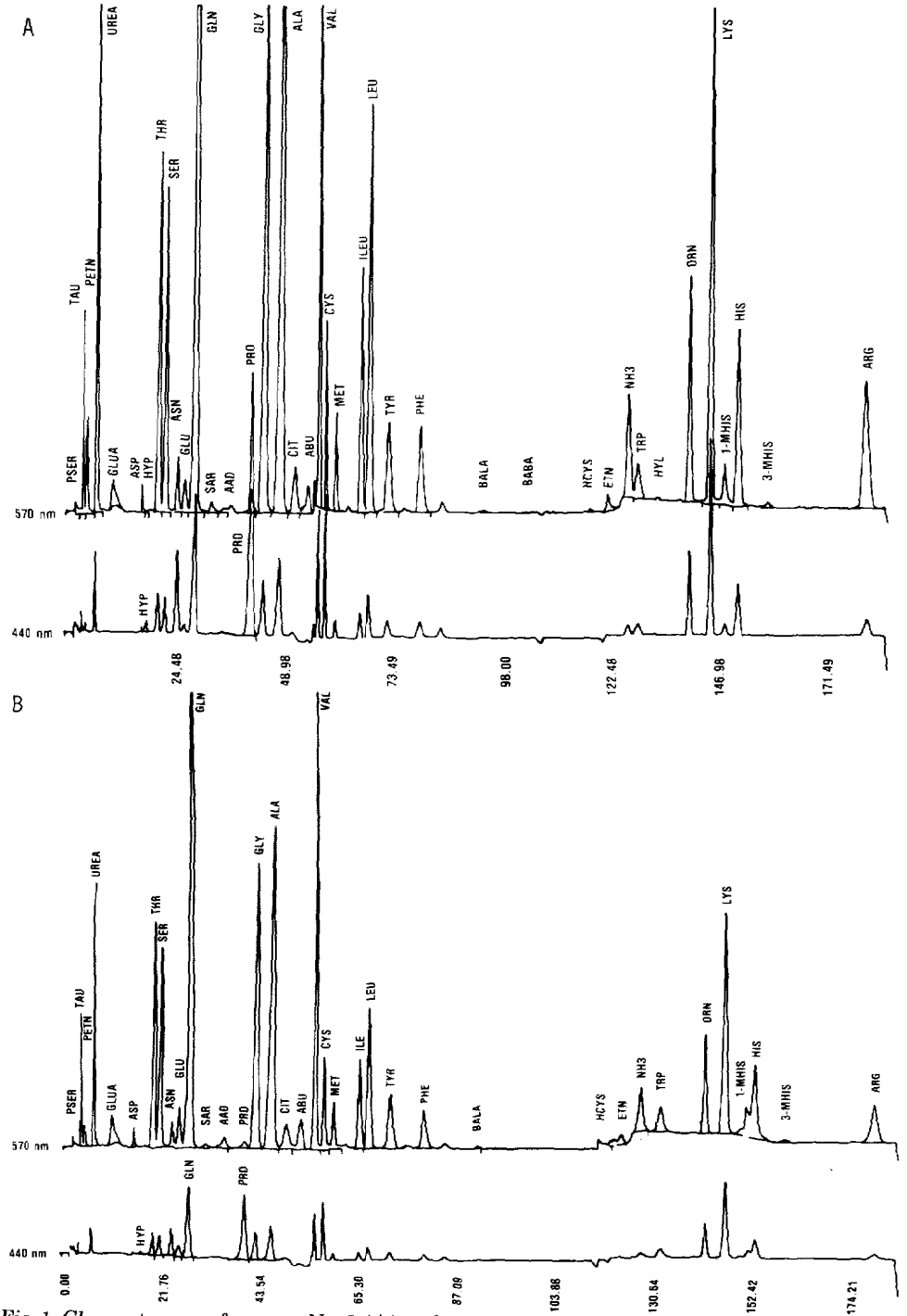

Fig. 1. Chromatograms from run No. 9 (A) and run No. 1081 (B), showing the two recorder tracings (570 and 440 nm), of the free amino acids from two different EDTA normal plasma samples deproteinized with 3.5 mg of SSA per 100 μl of plasma, and Centrifree ultrafiltration, with GLUA (glucosaminic acid) added as a quality control standard. IUB-IUPAC Joint Commission on Biochemical Nomenclature abbreviations are used where possible.

This method includes a rapid preparation of sample from freshly drawn blood which is preferably cooled prior to and during centrifugation to minimize metabolic changes in free amino acids; EDTA rather than heparin is used as the anticoagulant to provide a pH of 1.8–1.9, in combination with a concentration of SSA (3.5 mg per 100 μl of plasma); along with an adjustment of the pH with Li-S buffer to a pH of 2.0–2.2 after protein is removed. This procedure has effectively removed the shifts in amino acid peaks and the artifacts due to excess SSA while providing maximum recoveries of free amino acids in plasma. The use of D-glucosaminic acid as a quality control standard is certainly needed for clinical work. Unfortunately, some 15% of plasma samples have an unknown amino acid(s) in this same position, so we expect our glucosaminic acid values to be usually higher by 3–4 ng/ml than what we have added. This serves our purpose, while we look further for that perfect internal quality control standard.

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