ANALYTICAL BIOCHEMISTRY 82, 236–239 (1977)

SHORT COMMUNICATIONS

A New Method for Deproteinization of Small Samples of Blood Plasma for Amino Acid Determination[1]

In this work is described a simplified plasma deproteinization procedure using acetone and very small amounts of plasma. The precipitation is carried out in small-bore capillary tubes, and acetone is used as deproteinizing agent. This method can be advantageously compared with heat, Somogyi's, perchloric, and even trichloroacetic deproteinization procedures for its good amino acid preservation and protein removal capabilities.

In order to obtain plasma extracts free of protein as a first step in the determination of free amino acids by means of a Dansyl chloride estimation technique (1–3), we faced the problem imposed by very small amounts of sample available for deproteinization. The usual methods require a considerable volume and the action of strong agents that can produce alterations of the amino acids present in the sample (e.g., tryptophan, glutamine, etc.); if these requirements are not met, some of the materials are carried in the precipitate, giving low recoveries. A good approximation to our goal is the method described by Briel and Neuhoff (2) that uses deproteinization with acetone in the cold. The problem of handling minute plasma samples in the order of 5–25 μl, as was available in our sequenced studies, remained.

This goal has been accomplished by using capillary tubes, which leave practically all the amino acids in the supernatant and thereby give a very good recovery of labeled amino acids in the supernatant.

MATERIALS AND METHODS

Plasma from adult Wistar rats was obtained from heparinized blood. Amino acids labeled with ^{14}C were purchased from Amersham/Searle: L-[U-^{14}C]alanine, 10 mCi/mmol; L-[U-^{14}C]asparagine, 100 mCi/mmol; L-[U-^{14}C]-aspartic acid, 208 mCi/mmol; DL-[3-^{14}C]cysteine, 25 mCi/mmol; L-[U-^{14}C]arginine, 150 mCi/mmol; L-[U-^{14}C]glutamic acid, 249 mCi/mmol; [U-^{14}C]glycine, 50 mCi/mmol; L-[ring-^{14}C]histidine, 57.8 mCi/mmol; L-[U-^{14}C]leucine, 4.63 mCi/mmol; L-[*methyl*-^{14}C]methionine, 25 mCi/mmol; L-[U-^{14}C]threonine, 100 mCi/mmol; L-[U-^{14}C]lysine HCl, 150 mCi/mmol; L-[U-^{14}C]tyrosine, 225 mCi/mmol; and L-[U-^{14}C]valine, 125 mCi/mmol. Trichloroacetic

[1] Work supported in part by a grant from the "Comisión Asesora para la Investigación Científica y Técnica" from the "Presidencia del Gobierno" of Spain.

Copyright © 1977 by Academic Press, Inc.
All rights of reproduction in any form reserved.

ISSN 0003-2697

54

TABLE I

PERCENTAGE CONCENTRATION OF ADDED ^{14}C-LABELED AMINO ACIDS IN THE SUPERNATANTS (VOLUME CORRECTED) AFTER PLASMA ACETONE PRECIPITATION

^{14}C-Labeled amino acid	Mean recovery[a]
Arginine	87.8 ± 1.2
Valine	92.4 ± 10.4
Lysine	93.5 ± 1.1
Glycine	99.1 ± 3.1
Aspartic acid	99.6 ± 0.1
Asparagine	101.2 ± 1.0
Leucine	101.6 ± 1.0
Methionine	101.7 ± 1.6
Serine	102.3 ± 0.5
Tyrosine	102.5 ± 1.3
Histidine	102.6 ± 1.4
Phenylalanine	103.3 ± 0.5
Glutamic acid	103.3 ± 2.0
Threonine	103.8 ± 0.4
Alanine	105.7 ± 0.8
Mean	100.03 ± 0.54

[a] Mean recovery ± sem of six to eight determinations expressed as the percentage of added initial radioactivity.

acid (TCA) and acetone (both from Carlo Erba, Milano) were of analytical reagent quality. Narrow-bore capillary tubes, 100 mm long with an internal diameter of approximately 0.565 mm, were purchased from Radiometer (Copenhagen). All glassware was washed with 0.1 N NaOH and concentrated NO_3H according to Lowry and Passoneau (4). Samples were counted with a PPO/POPOP toluene/Triton X-100-based cocktail (5) in a Nuclear Chicago Isocap 300 scintillation counter, correcting the quenching of the samples with an external standard ratio device.

RESULTS AND DISCUSSION

Plasma samples from 2 to 25 μl were deproteinized with 1.4 vol of acetone in capillary tubes. The tubes were maintained horizontally on a strong wire support, and plasma was pipetted into one of the open ends of the tube. Acetone was pipetted into the other open end, and this side was plugged with sealing paste (Cryto-Seal, Fisher). The tubes were then placed in a vertical position, with the acetone and the plugged end down, and were mixed by means of a short centrifugation (2 min at 500g) taking advantage of the difference in density for a thorough mixing. Immediately, a precipitate appeared along the entire tube length. The tubes were then left to settle in upright position at −30°C overnight (or a minimum of 4 h)

and then were centrifuged for 20 min at 1500*g* in the cold. A clear supernatant appeared, and a very definite precipitate of protein was separated. Supernatant and precipitate can be easily separated by means of an incision with a file and the breaking of the tube. In this way very little supernatant is lost. The precipitate end of the tube can be saved for radioactivity evaluation or for protein determinations.

The recovery values for ^{14}C-labeled amino acids added to the plasma before deproteinization with acetone are shown in Table 1. It can be seen that the recoveries are very constant, and the only amino acids that seem to be partly trapped in the precipitate are arginine, valine, and lysine; but, in none of these amino acids can there be shown significant differences with respect to the means of the other amino acids. The precipitation with acetone does not require any further maneuvers, and the supernatants can be used directly for reaction with Dansyl chloride or fluorescamine.

A similar procedure was conducted, substituting the acetone with 2 vol of 10% (w/v) TCA (this concentration was tested as the lowest that precipitated all protein in our plasma samples). Two procedures were tested. In the first, the TCA supernatants were dried *in vacuo* and the dry residues were washed three times with anhydrous ethyl ether. It was found that the procedure for TCA extraction paid a significant toll in the total amino acids recovered in the dry residue, as 35.9% of the supernatant amino acids were lost in the combined ether washings. The other procedure tested involved the repeated extraction of the TCA supernatant with water-saturated ethyl ether. The recoveries of radioactive amino acids added to plasma samples were better, as now the amino acid loss in the three combined ether washings was only 21.9%. Additionally, the procedure was considerably cumbersome, and TCA has been found to interfere with the dansylation procedure, due to its acidity. A concentration of 5 mM TCA in the sample caused a mean 15.6% inhibition in the dansylation of a mixture of 22 amino acids; this concentration is about one hundredth of the initial TCA concentration in the supernatant. A 10-fold increase in the TCA concentration, up to 50 mM, gave 72.2% inhibition; with 500 mM TCA, the inhibition was greater than 98.5%. When neutralized carefully with NaOH, TCA showed no appreciable effect upon the dansylation procedure, but the excess of salts proved to be a hindrance in sample deposition on the chromatographic plates. Small amounts of TCA did not interfere with the fluorescamine reaction, due to the large excess of buffer in the determination conditions.

The recoveries of added radioactive amino acids in plasma samples deproteinized with TCA in capillary tubes were of the same order of magnitude as those of acetone supernatants: 97.6% for a mixture of arginine, leucine, and threonine and 93.5% for glutamic acid, alanine, leucine, histidine, and phenylalanine.

We have tested the acetone deproteinization procedure against the usual perchloric acid deproteinization, followed by potassium bicarbonate neutralization, and have obtained larger yields in total fluorescence with

either Dansyl- chloride or fluorescamine when using the perchloric acid technique rather than acetone deproteinization, indicating heavy protein or peptidic interference. Thin-layer chromatography of Dansyl derivatives of perchloric acid supernatants, carried out according to Brown and Perham (3) and Snodgrass and Iversen (6), showed very considerable fluorescent Dansyl-protein interference in the plates. Protein determination in these supernatants showed a slight (1.2%) but appreciable protein contamination that did not precipitate with perchloric acid. The Somogyi supernatants were of no practical use in amino acid determination because the Somogyi precipitation (7) is known to adsorb several amino acids in its small precipitate particles.

Furthermore, we have found the presence of relevant amounts of fluorescamine and Dansyl chloride reacting materials that are acetone- or TCA-precipitable in the Somogyi supernatants. We found that using heat as a deproteinizing agent (boiling the samples for 2 min), was not sufficiently effective because some TCA- or acetone-precipitable fluorescamine reacting materials remained in the boiled supernatant together with the amino acids.

The recuperation check carried out with the acetone precipitation procedure described here show that it is indeed a very useful and handy routine for deproteinizing small quantities of plasma for sensitive identification reactions, and that there is not any definite trend toward the disappearance of any amino acid group. The use of capillary tubes suggested here as the place for deproteinization makes possible the use of very small amounts of plasma with maximal recovery.

REFERENCES

1. Seiler, N. (1970) *Methods Biochem. Anal.* **18**, 259–337.
2. Briel, G., and Neuhoff, V. (1972) *Hoppe Seyler's Z. Physiol. Chem.* **353**, 540–553.
3. Brown, J. P., and Perham, R. N. (1973) *Eur. J. Biochem.* **39**, 69–73.
4. Lowry, O. H., and Passoneau, J. V. (1972) A Flexible System of Enzymatic Analysis, Academic Press, New York.
5. Turner, J. C. (1971) Sample Preparation for Liquid Scintillation Counting, Amersham–Searle, Arlington Heights, Ill.
6. Snodgrass, S. R., and Iversen, L. L. (1973) *Nature New Biol.* **241**, 154–156.
7. Somogyi, M. (1945) *J. Biol. Chem.* **160**, 69–76.

L. Arola
E. Herrera[2]
M. Alemany

Cátedra de Fisiología General
Facultad de Biología
Universidad de Barcelona
Barcelona 7, Spain
Received December 5, 1975; accepted May 16, 1977

[2] To whom all correspondence should be addressed.