AN IMPROVED METHOD OF PLASMA DEPROTEINATION WITH SULPHOSALICYLIC ACID FOR DETERMINING AMINO ACIDS AND RELATED COMPOUNDS

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SUMMARY

After critically evaluating some plasma deproteination procedures involving the use of sulphosalicylic acid reported in the literature, a new method is proposed in which this protein precipitating agent is used that is particularly suitable when lithium buffers are used as resin elutriants. By buffering the deproteinating medium at pH 1.8, the final pH of the test solution becomes very close (without any additional adjustment) to that of the calibrating solution. In this way, the resolution in the earlier regions of chromatograms is not impaired when lithium systems are used for determining neutral and acidic amino acids. The amino acid recovery has been experimentally tested and found to be excellent for all the amino acids and related compounds except tryptophan, for which it was less than 94%. A scintillation technique with tritium-labelled lysine has also been used in order to check the validity of the proposed method.

INTRODUCTION

The pH of the test solution applied to a resin column for the automatic chromatography of amino acids and related compounds can have a considerable effect on the elution times and peak fraction volumes of some acidic amino acids, particularly when lithium buffers are used for eluting the resin column, as reported by MONDINO in a previous paper[1]. Moreover, MONDINO et al.[2] have recently demonstrated how, in a lithium cycle, variation of the pH of the feed pulse can cause dramatic displacements of the aspartic acid peak, which can seriously affect its separation from threonine and hydroxyproline. Consequently, from this point of view, a deproteination method, which alters the pH of the sample test solution relative to that of the calibrating solution, must be reconsidered when lithium buffers (which are the only ones suitable for the separation of glutamine and asparagine normally found in plasma and biological samples) are used as resin elutriants.

It should be advisable that, whatever protein precipitant is used, the excess of the deproteinating acid (picric, sulphosalicylic, trichloroacetic or tungstic acid) were removed by passing the supernatant liquid through a column of cation-exchange resin.

In this way, the ammonia eluate from this column, containing the amino acids, can be evaporated to dryness and then redissolved in a medium having the same pH as the calibrating solution. But, as already stated by other investigators[3] in criticism of the picric acid method of STEIN AND MOORE[4], such a procedure is time-consuming and errors may arise because of the many manipulations involved. The use of a resin column for removing the protein precipitating acid may involve, during the loading cycle, some losses of acidic amino acids, the leakage of which from the column is difficult to control. Then, during elution with an ammonia solution, some losses of basic amino acids, such as arginine, can occur owing to the difficulty of liberating them from the resin.

This effect may be the explanation of the low recoveries of aspartic acid, threonine, serine and arginine obtained by GERRITSEN et al.[5] using the ultracentrifugation method of deproteination. Moreover, in this method of protein removal, the pH of the plasma sample must be adjusted to a value that does not disturb the chromatographic equilibrium of the long column; this is not always possible owing to the small amounts of sample that are usually available and, moreover, such manipulation may be a source of error or inaccuracy.

HAMILTON[6], when using sulphosalicylic acid as a precipitating agent, removed plasma proteins by adding 0.5 ml of a 3% aqueous solution of the acid to 0.1 ml of plasma, but, as the pH of the supernatant liquid obtained in this way can reach about 1.3, this method is not suitable when lithium buffers are used as column elutriants.

For the same reason, the method proposed by BLOCK et al.[3], who prepared the plasma sample by adding 1 ml of a 20% aqueous solution of sulphosalicylic acid and 1 ml of water to 2 ml of plasma, can alter the first part of the chromatogram when the resin is eluted with lithium buffers; the pH of the test solution reaches about 1.2, so that the separation of the threonine–serine pair as well as the glutamic acid–glutamine pair is no more acceptable and the separation of aspartic acid from threonine becomes incomplete.

Our experience is in agreement with that of PETERS AND BERRIDGE[7], who reported that deproteinized samples containing sulphosalicylic acid as prepared by HAMILTON[6] gave poor resolution in the earlier regions of chromatograms when the lithium system was used for determining neutral and acidic amino acids.

The deproteination method used by GERRITSEN et al.[5], involving the use of a 4.5% aqueous solution of sulphosalicylic acid, cannot be accepted. In their method, the precipitate that is obtained by adding 4 ml of precipitating solution and 1 ml of internal standard solution to 1 ml of plasma must be washed with 0.1 N hydrochloric acid; such an operation causes a partial redissolution of the proteins. When the two supernatants are mixed, reprecipitation of proteins is observed and the resulting cloudy solution cannot be suitable for loading into the column; furthermore, the method prescribes a pH adjustment after the hydrochloric acid washing. All these manipulations can obviously be sources of errors and inaccuracy.

In a preceding paper, MONDINO[1] proposed to remove plasma proteins by employing 30 mg of lyophilized sulphosalicylic acid per millilitre of plasma. It was observed (if only 0.2 ml of the supernatant was loaded on to the column) that very good reproducibility of the elution times of the acidic amino acids of the sample in comparison with those of the calibration mixture could be attained.

We have observed that this method of removing proteins increases the concen-

tration of the remaining solutes because of a diminution of the sample volume, with a consequent alteration of the accuracy of the plasma amino acid determination. From a study of the recovery of amino acids and related compounds added to plasma samples before deproteination, it was found that the experimental results had to be multiplied by a factor of 0.9 in order to give the true value.

In this paper, previously adumbrated[2], we describe a method of using sulphosalicylic acid as a deproteinating agent that satisfies the pH conditions of the test solution relating to the lithium buffer requirements and overcomes the above difficulties associated with the previously proposed method[1].

An experimental study on the recoveries of amino acids and related compounds, usually found in plasma, by the new method is reported; for all of them, except tryptophan, the recoveries are close to 100%. The recoveries relate to amounts of amino acids and related compounds added to fixed volumes of previously analyzed human plasma. The analyses are carried out by methods involving the use of lithium buffers for both long and short columns as reported in previous papers[1,8].

In order to confirm these findings by another method of analysis, the recovery of [3H]lysine added to plasma samples, subsequently deproteinated by the new method, was studied. The results were satisfactory.

TABLE I

PUMP FILLING AND SETTING SCHEDULE

Pump No.	Filling	Flow-rate (ml/min)
1	Colour reagent	0.5
2	Buffer, pH 2.8	1
3	Buffer, pH 4.15	1
4	0.3 N LiOH	1
5	Buffer, pH 2.8	1
6	Colour reagent	0.5
7	Buffer, pH 4.52	1

EXPERIMENTAL

Apparatus and materials

Amino acid analyzer. An apparatus for automatic amino acid analysis described by MONDINO[9] and manufactured by Optica Co., Milan, equipped with seven pumps, was used. The pumps were filled as indicated in Table I, which also gives the relative flow-rates.

Pumps Nos. 1–5 operated the long column channel, and pumps Nos. 6 and 7 operated the short column channel that separates the basic amino acids and related compounds. Pump No. 2 was pre-set to pump 190 ml of buffer of pH 2.80 and then to stop after having started pump No. 3. Pump No. 3 was pre-set to pump 100 ml of buffer of pH 4.15 and then to stop. The temperature of the columns was then lowered to T_1 (37.5°) from T_2 (55°) and pump No. 4 was started and pre-set to pump 50 ml of 0.30 N lithium hydroxide and then to stop after having started pump No. 5, which pumped 120 ml of buffer of pH 2.80 in order to automatically equilibrate the long column resin for the next analysis. Washing and equilibration of the short column was

accomplished before the merging of proline in channel No. 1 as already described[8]. The operation of pumps Nos. 6 and 7, including their filling and flow-rate setting, has been described earlier[8].

Columns and resins. For the separation of the acidic and neutral amino acids and related compounds, an Amberlite IR-120 crushed resin was used; it was prepared in our laboratory as described in previous papers[2,9,10]. This resin was contained in a Pyrex glass column of total length 50 cm and I.D. 1 cm; the resin bed was 42 cm high, so that the apparent resin volume was 33 ml. The column temperature was maintained at 37.5° for 144 min and then increased to 55° for the remainder of the analysis.

For the separation of basic amino acids and related compounds, an Aminex A5 spherical resin was used. The conditions used for the glass column, the resin bed and the operating temperature were as reported earlier[8].

The operating pressure of the long column did not exceed 11 kg/cm² and that of the short column was about 6 kg/cm².

Photometers and recorders. The sensitivity of the photometer used for measurements on the long column eluate at 570 nm was set at 1 O.D. for full-scale deflection of the recorder pen, and the sensitivity of the second colorimeter used for measurements on the long column eluate at 440 nm was set at 0.5 O.D. After the elution of proline, the sensitivity of the second photometer (now re-set at 570 nm) was lowered to 1 O.D.

The optical path of the three flow-cuvettes was 10 mm long.

The speed of the chart on the two-strip chart solid-line recorders was set at 3 in./min.

Lithium buffers and lithium hydroxide solution. The compositions and the method of preparation of these solutions for the operation of both columns have been described in previous papers[1,8].

Colour reagent. The ninhydrin solution was prepared according to the method described by MONDINO in previous papers[1,9].

Buffer of pH 5.5 for the colour reagent. This was prepared according to the method of SPACKMAN et al.[11].

Calibrating solution. Amino acids and related compounds obtained from Fluka (Buchs F.G., Switzerland) were dissolved in 0.3 N lithium citrate buffer of pH 2.2. One millilitre of the final solution contained 50 nmole of each amino acid or related compound, except cystine and taurine, the concentration of which was 25 nmole/ml.

Aqueous solution of amino acids and related compounds. An aqueous solution containing all the amino acids and related compounds present in the calibrating solution was prepared. One millilitre of this solution contained 200 nmole of each amino acid or related compound, except cystine and taurine, the concentration of which was 100 nmole/ml.

Human blood plasma. The samples of human blood plasma were prepared by centrifuging at 3500 r.p.m. (after addition of sodium EDTA). Blood samples were obtained from volunteers.

Deproteinating solution. A 3.75% sulphosalicylic acid solution in 0.3 N lithium citrate buffer of pH 2.8 was prepared. The pH of this solution was adjusted to 1.8 by adding a few drops of a solution of concentrated lithium hydroxide.

Liquid scintillation spectrometer. A Liquid Scintillation System 720 manufactured by Nuclear Chicago Co. (Des Plaines, Ill., U.S.A.) was used.

[³H]Lysine solution. A solution of tritiated L-lysine with an activity of 175 μCi/ml and a lysine concentration of 125 nmole/ml was obtained from CEN (Belgium).

Digestion solvent. NCS (Nuclear Chicago Solvent) digestion solvent was purchased from Nuclear Chicago Co. (Des Plaines, Ill., U.S.A.).

PPO, POPOP* and scintillation-grade toluene. These solvents were purchased from Merck (Darmstadt, G.F.R.).

Tritium-quenched standards. Tritium standards of various efficiencies caused by a different degree of quenching, but containing known amounts of radioactivity, were purchased from Nuclear Chicago Co. (Des Plaines, Ill., U.S.A.).

METHODS

The deproteination of plasma was achieved by adding 4 ml of the deproteinating sulphosalicylic acid solution to 1 ml of plasma exactly measured into a Servall No. 201 "Wasserman" centrifuge test-tube. The mixture was shaken for a few seconds and then centrifuged at 10,000 r.p.m. for 10 min at 0° in order to avoid any possible amino acid alteration. One millilitre of supernatant liquid was loaded on to each column for the determination of amino acids and related compounds.

In order to ascertain whether this deproteination method interferes with the accuracy of the amino acid determination, amino acids and related compounds were added to another sample of the same plasma, which was then deproteinated and analyzed. The addition was performed in the following way: 2 ml of the aqueous solution of amino acids and related compounds were evaporated to dryness in a Büchi rotoevaporator equipped with a 25-ml rotary flask at room temperature. Then 2 ml of plasma were introduced into the flask, which was allowed to rotate at room temperature (without vacuum) for 15 min in order to obtain the complete dissolution into the plasma of the dried amino acids and related compounds contained in the flask. One millilitre of this amino acid-containing plasma was deproteinated with 4 ml of the deproteinating sulphosalicylic buffer as previously described and then analyzed as before in order to calculate the recoveries of the added amino acids. The chromatograms of both the plasma and the amino acid-containing plasma samples were calibrated against chromatograms obtained by loading 1 ml (50 nmole) of the calibrating solution on to each column. The chromatograms were evaluated by measuring the height of peaks over the base-line in millimetres, as in our analytical system this method of peak digitization is possible and, as reported in preceding papers[1,0,12], it gives greater precision than that of the method of $H \times W$ integration, in as much as the repeatability from standard chromatogram to sample chromatogram is extremely good[2].

The number of milligrams of each amino acid per 100 ml of plasma was obtained from the following equation:

$$\text{Amino acid (mg per ml of plasma)} = \frac{H \times nM \times MW}{H_S \times V_p \times 10^4}$$

where

H = peak height (mm) of the amino acid on the chromatogram.

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* PPO = 2,5-diphenyloxazole; POPOP = *p*-bis-2-(5-phenyloxazolyl)-benzene.

nM = number of nmoles of the amino acid that have given H_S on the standard chromatogram.

MW = molecular weight.

H_S = peak height (mm) of the amino acid on the standard chromatogram.

V_p = Volume of plasma loaded on the column (ml), calculated by dividing the volume of the deproteinated plasma solution applied to the column by the dilution factor.

Furthermore, the recovery of a measured amount of [³H]lysine added to a plasma sample, before removing proteins by the proposed method, was determined by the scintillation counting technique. In this method, 50 μl of the [³H]lysine solution, having an activity of 175 μCi/ml, were added to 4 ml of human plasma. One 1-ml aliquot of this preparation was deproteinated and centrifuged as described above. A second 1-ml aliquot of the same preparation was diluted with 4 ml of lithium citrate buffer of pH 2.2. Then 0.5 ml of both the deproteinated and diluted plasma were allowed to digest at room temperature with 3 ml of NCS solvent for 30 min in two scintillation glass vials. In parallel, 50 μl of an unlabelled lysine solution, having the same concentration of lysine as the labelled solution, were added to 4 ml of the same human plasma. From this preparation, two samples were prepared and digested by the same procedure as before, in order to obtain the background rates for the corresponding samples containing the radioactive tracer. Then, to each vial, 10 ml of the scintillation solution (PPO, POPOP in toluene) and 6.5 ml of toluene were added in order to obtain final concentrations of 6 g/l of PPO and of 50 mg/l of POPOP. After cooling for 24 h at 4° in the spectrometer refrigerator, the radioactivity of the vial contents was measured, and the recoveries were calculated. The determination of the counting efficiency of liquid scintillation plasma samples, in order to obtain the number of disintegrations per minute (d.p.m.), was performed according to the method of BUSH[13] as a function of the ratio of the count-rates in two channels of the pulse height spectrum. A calibration curve was established by counting a series of tritiated samples of various efficiencies (degrees of quenching), but containing the same known amount of radioactivity.

RESULTS AND DISCUSSION

Table II summarizes the results of the recovery test performed on four plasma samples obtained from four different donors. The values reported are the percentage ratios between the concentrations determined in the amino acid-containing plasma and the sum of the amino acid concentrations determined in the same plasma before the addition plus the theoretical value of the amounts of amino acids added. They are therefore the determined results as percentages of the expected values. Mean and standard deviations are also reported.

The recoveries of all the amino acids and related compounds, except tryptophan approach 100%. Therefore, if sulphosalicylic acid is used efficiently as the deproteinating agent, no significant losses occur for all the amino acids except tryptophan, the recovery of which is less than 94%. In this aspect we do not agree with PETERS AND BERRIDGE[7], who stated that loss of tryptophan can be avoided by using sulphosalicylic acid to precipitate proteins from plasma samples. However, in our laboratory, studies are being made in order to solve this problem.

TABLE II

RECOVERY TEST PERFORMED ON FOUR PLASMA SAMPLES FROM DIFFERENT DONORS

The values reported were calculated according to the following equation:

$$\text{Recovery } (\%) = \frac{a}{p + t} \times 100$$

where a is the experimentally determined concentration in amino acid-containing plasma, p is the amino acid concentration found in the same plasma without addition of amino acids, and t is the theoretical value of the amount of amino acid added to the plasma.

Sample	1	2	3	4	Mean	S.D.
Taurine	101.1	101.5	100.5	103.7	101.7	1.39
Aspartic acid	99.2	99.1	99.2	100.8	99.5	0.81
Threonine	96.3	100.0	97.8	102.6	99.1	2.74
Serine	98.2	100.5	99.1	98.4	99.0	1.04
Asparagine	100.2	100.0	102.8	100.2	100.8	1.33
Glutamic acid	100.0	99.5	98.8	99.8	99.5	0.52
Glutamine	101.3	102.2	102.4	101.2	101.7	0.61
Proline	100.7	101.2	102.3	100.9	101.2	0.71
Glycine	100.9	99.4	100.9	96.6	99.4	2.02
Alanine	99.4	99.1	101.7	98.9	99.7	1.29
Citrulline	99.7	100.4	99.5	99.7	99.8	0.39
AABA[a]	99.1	98.3	99.0	99.1	98.8	0.38
Valine	98.3	100.0	102.2	104.9	101.3	2.85
Cystine	98.5	100.0	99.1	98.7	99.0	0.66
Methionine	102.4	102.1	98.4	99.7	100.6	1.92
Isoleucine	101.9	100.0	100.8	99.4	100.5	1.08
Leucine	100.7	99.5	100.0	98.1	99.5	1.09
Tyrosine	100.0	101.8	100.0	99.6	100.3	0.98
Phenylalanine	100.4	101.5	100.9	100.3	100.7	0.55
Tryptophan	93.2	96.3	92.2	94.3	94.0	1.75
Ornithine	101.7	103.2	99.4	101.0	101.3	1.57
Lysine	102.7	102.2	99.02	99.5	100.8	1.86
Histidine	99.2	101.5	100.4	99.5	100.1	1.03
Arginine	103.0	100.4	98.9	100.4	100.6	1.70

[a] AABA = α aminobutyric acid.

This method of using sulphosalicylic acid as a deproteinating agent can overcome possible sources of artifacts that may occur when this acid is used according to the various methods described in the literature, especially when lithium buffers are used as resin elutriants.

It can be used with advantage because, together with the protein precipitation, an optimum pH of the amino acid test solution is obtained (pH 2.2, which is the pH of the calibrating solution) without any further dilution or manipulation. In fact, the precipitation of the proteins involves, and then removes from the precipitating solution, part of the dissolved sulphosalicylic acid, which is precipitated with the proteins. Consequently, the pH of the precipitating buffer, which is initially about 1.8, increases to about 2.2.

For this reason, the value of the pH of the precipitating buffer of 1.8 is valid for all plasma samples that contain normal or about normal amounts of plasma proteins. It was observed that in a case of agammaglobulinaemia, it would have been advisable to increase the pH of the deproteinating buffer solution of sulphosalicylic acid.

Another advantage of this method is that it overcomes the problem of the decrease in the volume of plasma following protein removal, which can become impor-

TABLE III

DETERMINATION OF [³H]LYSINE ADDED TO PLASMA WITH AND WITHOUT DEPROTEINATION

Mean efficiency for tritium = 18.6%.

Plasma sample	d.p.m.	Differences	Ratio (%)
I	1,972,340[a]	1,972,205	100.1
	135[b]		
	1,974,547[c]	1,974,394	
	153[d]		
2	1,940,800[a]	1,940,635	101.8
	165[b]		
	1,975,742[c]	1,975,590	
	152[d]		
3	1,957,960[a]	1,957,847	101.1
	113[b]		
	1,980,191[c]	1,980,075	
	116[d]		

[a] Non-deproteinated plasma.
[b] Background of non-deproteinated plasma.
[c] Deproteinated plasma.
[d] Background of deproteinated plasma.

tant when solid sulphosalicylic acid is used, as reported earlier in this paper. This is virtually avoided owing to the 1:5 dilution of the plasma sample, and is also confirmed by the experiment concerning the deproteination of a sample containing a known amount of [³H]lysine.

Table III summarizes the results of this experiment, which was performed on three different plasma samples as previously described. It can be seen that there is no appreciable difference between the d.p.m. values counted for the tritiated lysine in the non-deproteinated plasma solution and those in the deproteinated samples according to the method described here.

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REFERENCES

1 A. MONDINO, J. Chromatogr., 39 (1969) 262.
2 A. MONDINO, G. BONGIOVANNI AND S. FUMERO, J. Chromatogr., 71 (1972) 363.
3 W. D. BLOCK, M. E. MARKOVS AND B. F. STEELE, Proc. Soc. Exp. Biol. Med., 122 (1966) 1089.
4 W. H. STEIN AND S. MOORE, J. Biol. Chem., 211 (1954) 915.
5 T. GERRITSEN, M. L. REHBERG AND H. A. WAISMAN, Anal. Biochem., 11 (1965) 460.
6 P. B. HAMILTON, Ann. N.Y. Acad. Sci., 102 (1962) 55.
7 J. H. PETERS AND B. J. BERRIDGE, JR., Chromatogr. Rev., 12 (1970) 157.
8 A. MONDINO, G. BONGIOVANNI, V. NOE AND I. RAFFAELE, J. Chromatogr., 63 (1971) 411.
9 A. MONDINO, J. Chromatogr., 30 (1967) 100.

10 A. MONDINO, *J. Chromatogr.*, 50 (1970) 260.
11 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
12 A. MONDINO, *J. Chromatogr.*, 41 (1969) 156.
13 E. T. BUSH, *Anal. Chem.*, 35 (1963) 1024.