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# A NEW APPROACH FOR OBTAINING TOTAL TRYPTOPHAN RECOVERY IN PLASMA SAMPLES DEPROTEINIZED WITH SULPHOSALICYLIC ACID

ANGELO MONDINO, GIANNI BONGIOVANNI and SILVANO FUMERO
*Istituto di Ricerche Biomediche Antoine Marxer S.p.A., R.B.M., 10015 Ivrea (Italy)*
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## SUMMARY

The treatment of plasma with a sodium dodecylsulphate solution before deproteinization with sulphosalicylic acid is described. With this treatment, the liberation of tryptophan from plasma albumin is complete and its determination can be made directly, together with that of other amino acids and related compounds, by automatic ion-exchange chromatography, eliminating the need for an extra analysis for the accurate determination of this amino acid.

Results of tryptophan recovery tests, carried out both on plasma samples simply deproteinized with sulphosalicylic acid and on other samples pre-treated with sodium dodecylsulphate solution are reported. These results are also compared with those obtained when trichloroacetic acid was used as deproteinizing agent.

A recovery study on a human albumin (fraction V) solution containing a known amount of tryptophan is also described.

## INTRODUCTION

As reported previously[1–4], tryptophan occurs in plasma in two forms: free and bound to plasma albumin. It has also been demonstrated by Opieńska-Blauth *et al.*[5] that the carboxyl group of the tryptophan molecule is responsible for its binding with the active sites of the protein molecule. As treatment with trichloroacetic acid renders this binding labile[5], this deproteinizing agent is commonly used in preparing plasma samples on which a tryptophan determination is to be effected fluorimetrically[6,7].

In contrast, other protein precipitants, such as picric acid and sulphosalicylic acid (usually employed for preparing plasma samples for the determination of amino acid and related compounds by ion-exchange chromatography), are incapable of liberating this amino acid quantitatively from plasma proteins.

Stein and Moore[8] and Dickinson *et al.*[9] reported that partial losses of plasma tryptophan occur when picric acid is used as deproteinizing agent. Mondino *et al.*[10] also recently demonstrated that, using a novel sulphosalicylic acid plasma deproteinization procedure (particularly suitable when lithium buffers are employed for resin elution), the recovery was excellent for all of the amino acids and related compounds

considered, except tryptophan, for which it was less than 94%. Gerritsen *et al.*[11] also obtained erratic results for tryptophan when removing the plasma proteins by ultracentrifugation.

Consequently, up to now, when very accurate values of plasma tryptophan were sought, together with those of the remaining amino acids, it has been necessary to follow two types of analytical procedures. The aim of our research was to find a plasma sample processing method that would allow the simultaneous quantitative determination of all of the amino acids and related compounds, including tryptophan using automatic ion-exchange column chromatography.

A treatment was devised whereby the plasma sample was subjected to an anionic protein denaturant which could, to a large extent, unfold the serum albumin molecule and, by strongly interacting with its active sites, displace the bound tryptophan without causing precipitation of protein. The detergent sodium laurylsulphate, the action of which on protein molecules was reported by Tanford[12], fulfilled these requirements. This denaturation procedure, followed by precipitation of protein with sulphosalicylic acid, as reported later in this paper, liberated tryptophan bound to serum albumin and hence permitted the complete recovery of this amino acid and also of the remaining amino acids. Furthermore, like sulphosalicylic acid, sodium laurylsulphate does not disturb either the amino acid chromatography or the ninhydrin colour reaction.

## EXPERIMENTAL

### *Apparatus*

An apparatus for automatic amino acid analysis described earlier by Mondino[13,14] and now manufactured by Optica (Milan, Italy) was employed. The flow-rates, columns and resin used, the photometer and recorder setting, lithium buffer composition, colour reagent and amino acid calibrating solution were the same as those employed in earlier work[15,16].

### *Materials*

*Aqueous tryptophan solution.* L-Tryptophan purchased from Fluka (Buchs, Switzerland) was used to prepare an aqueous solution of concentration 100 nmoles/ml.

*Human serum albumin.* Human serum albumin (fraction V) was purchased from the N.B. Co. (Cleveland, Ohio, U.S.A.).

*Human blood plasma.* The samples of human blood plasma, obtained from volunteers in our laboratories, were prepared by centrifugation at 2000 *g* (after addition of EDTA, sodium salt).

*Sodium dodecylsulphate (SDS) solution.* An aqueous solution containing 0.5 g of sodium dodecylsulphate (Fluka) per 100 ml was prepared.

*Sulphosalicylic acid (SSA) deproteinizing solution.* A 3.75% solution of sulphosalicylic acid in a 0.3 *N* lithium citrate buffer (pH 2.8) was prepared, as reported earlier[10]. The pH of this solution was adjusted to 1.8 by adding a few drops of concentrated lithium hydroxide solution.

*Trichloroacetic (TCA) solution.* A 10% aqueous solution of trichloroacetic acid (Merck, Darmstadt, G.F.R.) was prepared.

*Recovery test on plasma deproteinized with SSA*

A 4-ml volume of the 3.75% sulphosalicylic acid deproteinizing solution was added to 1 ml of a plasma specimen exactly measured into a Servall 201 "Wasserman" centrifuge tube. The mixture was shaken for a few seconds and then centrifuged at 15,000 *g* for 10 min at 0° in order to avoid any possible amino acid alteration. A 1-ml volume of the supernatant was loaded on to the short column for the determination of basic amino acids and related compounds. A 2-ml volume of the aqueous tryptophan solution was evaporated to dryness at room temperature in a Büchi rotary evaporator equipped with a 25-ml rotary flask. Then 2 ml of the same previously analyzed plasma specimen were introduced into the flask, which was rotated at room temperature (without vacuum) for 15 min in order to achieve the complete dissolution of the dried tryptophan into the plasma. A 1-ml volume of this plasma with tryptophan added was deproteinized with 4 ml of the 3.75% sulphosalicylic acid buffer, as previously described, and then analyzed as before in order to determine the tryptophan loss. The same operations were also performed by adding the dry content of 4 ml of the aqueous tryptophan solution to 2 ml of plasma.

*Recovery test on plasma pre-treated with SDS and deproteinized with SSA*

A 1-ml volume of a freshly prepared 0.5% aqueous solution of sodium dodecylsulphate was added to 1 ml of plasma in a Servall 201 "Wasserman" centrifuge tube. The mixture was allowed to stand for 15 min after shaking at room temperature and then 3 ml of the 5% sulphosalicylic acid deproteinizing buffer were added. The precipitation and removal of protein was performed as described above, in order to obtain a solution suitable for amino acid analysis on a short column. A 2-ml volume of the aqueous tryptophan solution was evaporated to dryness and the residue was added to 2 ml of plasma, as previously described. Then 1 ml of this tryptophan-enriched plasma was added to 1 ml of 0.5% aqueous sodium dodecylsulphate and deproteinized with 5% sulphosalicylic acid as described above. Tryptophan analysis was then performed by loading 1 ml of the clear solution (obtained after centrifugation) on to the resin column. The same operations were also performed by adding the dry content of 4 ml of the aqueous tryptophan solution to 2 ml of plasma.

*Comparison of tryptophan levels found in plasma samples deproteinized with SSA, with TCA or with SDS and SSA.*

Plasma samples from different normal, healthy individuals were treated in the following manner. A 1-ml volume of each sample was deproteinized with 4 ml of 3.75% sulphosalicylic acid deproteinizing solution as already described. A further 1 ml of the same plasma sample was deproteinized with 3 ml of 5% sulphosalicylic acid deproteinizing solution after addition of 1 ml of 0.5% sodium dodecylsulphate solution. A third 1 ml of the same plasma sample was deproteinized by addition of 4 ml of the 10% trichloroacetic acid solution. With all of these deproteinized solutions, the determination of tryptophan was performed by loading 1 ml of the centrifugates on to the short chromatographic column.

*Recovery study on human serum albumin*

In order to confirm that the binding of tryptophan to serum albumin is labile under the action of sodium dodecylsulphate, 1.5 ml of aqueous tryptophan solution

was diluted with 1.5 ml of water and then 150 mg of human serum albumin were added. The tryptophan: albumin ratio in this solution, and also their concentrations, were similar to those found in human plasma. This solution was maintained at 37° for a 2 h. A 1-ml aliquot of this solution was directly deproteinized, as previously described, with 4 ml of 3.75% sulphosalicylic acid solution and then 1 ml of the clear supernatant was subjected to tryptophan analysis. A second 1-ml aliquot was treated with 1 ml of 0.5% sodium dodecylsulphate solution, deproteinized with 3 ml of 5% sulphosalicylic acid solution and then analyzed as described above.

The evaluation of all the chromatograms was performed as described earlier[13–15].

## RESULTS AND DISCUSSION

Table I summarizes the results obtained on four plasma samples directly deproteinized with 3.75% sulphosalicylic acid without treatment with sodium dodecylsulphate. The tryptophan levels determined before addition of aliquots of this amino acid are reported in each instance. On two of them, 100 nmoles/ml (equal to 2.04 mg per 100 ml) were added; on the other two, 200 nmoles/ml (4.08 mg per 100 ml) were added; the results obtained by analyzing these samples containing added tryptophan are also reported.

TABLE I

TRYPTOPHAN (Trp) RECOVERY TEST ON HUMAN PLASMA SAMPLES DEPROTEINIZED WITH 3.75% SULPHOSALICYLIC ACID

| *Sample* | *A: Trp found (mg-%)* | *B: Trp added (mg-%)* | *C: A + B (mg-%)* | *D: value found (mg-%)* | *E: recovery (%)** | *F: recovery (%) of added Trp*** |
|---|---|---|---|---|---|---|
| 1 | 1.21 | 2.04 | 3.25 | 3.03 | 93.2 | 89.2 |
| 2 | 1.23 | 2.04 | 3.27 | 3.15 | 96.3 | 94.1 |
| 3 | 1.17 | 4.08 | 5.25 | 4.84 | 92.2 | 89.9 |
| 4 | 1.25 | 4.08 | 5.33 | 5.03 | 94.3 | 92.6 |

$$^{*}E = \frac{D}{C} \cdot 100.$$

$$^{**}F = \frac{D - A}{B} \cdot 100.$$

In the sixth column, the values reported are the percentage ratios between the tryptophan concentrations found in the plasma to which tryptophan was added and the sum of the tryptophan concentrations found in the same plasma before addition plus the theoretical value of the added amounts. In the seventh column, the values reported are the percentage ratios between the difference of the tryptophan concentration found in the plasma with tryptophan added minus the tryptophan concentration in the same plasma before addition and the theoretical value of the added amounts.

Considering these results, referred to the absolute recoveries of the amounts added, it can be assumed, in agreement with the findings of Opieńska-Blauth *et al.*[5],

that the plasma albumin molecules in the specimens examined still bore active sites available for tryptophan binding and that the tryptophan recoveries obtained from plasma deproteinized with sulphosalicylic acid are independent of the total amount of tryptophan present in the sample. In fact, the percentage recovery is of the same order in the plasma samples to which 2.04 mg per 100 ml of tryptophan was added as it is in those to which 4.08 mg per 100 ml was added.

Table II summarizes the results obtained on two plasma samples analyzed for tryptophan before and after the addition of different amounts of this amino acid. Before deproteinization, the samples were treated with sodium dodecylsulphate and, as can be seen, the recoveries of the added tryptophan closely approach 100%.

TABLE II

TRYPTOPHAN (Trp) RECOVERY TEST ON HUMAN PLASMA SAMPLES DEPROTEINIZED WITH 5% SULPHOSALICYLIC ACID PRE-TREATED WITH SODIUM DODECYLSULPHATE

| *Sample* | *A: Trp found (mg-%)* | *B: Trp added (mg-%)* | *C: A + B (mg-%)* | *D: value found (mg-%)* | *E: recovery (%)** | *F: recovery (%) of added Trp*** |
|---|---|---|---|---|---|---|
| 1 | 0.85 | 2.04 | 2.89 | 2.88 | 99.6 | 99.5 |
| 2 | 1.15 | 4.08 | 5.23 | 5.20 | 99.4 | 99.2 |

$$^{*}\,E = \frac{D}{C} \cdot 100.$$

$$^{**}\,F = \frac{D - A}{B} \cdot 100.$$

Concerning the determinations of tryptophan in human serum albumin with tryptophan added, the recovery obtained in the solution that was deproteinized without preliminary treatment with sodium dodecylsulphate was 93.7%. This result is in agreement with the values previously found in the recovery tests performed on human plasma. In contrast, the tryptophan recovery in the detergent-treated sample was 100.2%. This confirms the findings of Opieńska-Blauth *et al.*[5] on the capacity of albumin to bind tryptophan and also the lability of this binding under the action of the proposed detergent.

Table III shows the results obtained from nine plasma samples on which the tryptophan was determined after treatment with sulphosalicylic acid alone, with sodium dodecylsulphate and sulphosalicylic acid, and finally with 10% trichloro-

TABLE III

TRYPTOPHAN VALUES (mg-%) OBTAINED FROM PLASMA SAMPLES DEPROTEINIZED IN DIFFERENT WAYS

| *Agent* | *Sample No.* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* |
| 3.75% sulphosalicylic acid | 1.01 | 1.13 | 1.03 | 0.99 | 0.83 | 1.03 | 0.96 | 0.87 | 1.34 |
| SDS plus 5% sulphosalicylic acid | 1.08 | 1.19 | 1.08 | 1.02 | 0.87 | 1.05 | 1.18 | 0.94 | 1.43 |
| 10% trichloroacetic acid | 0.93 | 1.17 | 1.08 | 1 | 0.75 | 0.92 | — | — | — |

acetic acid. The assay with trichloroacetic acid was performed because this deproteinizing agent is commonly used, as already reported, in the fluorimetric determination of tryptophan, as the tryptophan–albumin binding is labile on deproteinization with this reagent. It was ascertained that this reagent does not interfere in the chromatographic separation of tryptophan in the short column, if it is not eliminated from the centrifuge supernatant, nor does it interfere in the colorimetric reaction with the ninhydrin colour reagent. Incidentally, it should be noted that this characteristic does not hold for all other amino acids.

From the results, it can be seen that the treatment of the plasma sample with sodium dodecylsulphate followed by deproteinization with sulphosalicylic acid gives the highest values for tryptophan. It should be mentioned that experiments were performed in order to ascertain whether, on increasing the amount of this detergent present, the recovery of tryptophan would also increase; this did not occur.

At the present state of our investigation, we feel that it would be advisable to introduce this variation when using sulphosalicylic acid as a protein precipitant because, this way, no extra analysis for the correct determination of tryptophan in plasma is necessary.

The pH of the centrifuge supernatant obtained with this procedure is about 2.2, that is the pH of the calibrating solution. Consequently, no correction is required before its application on to the chromatographic columns that are to be eluted with lithium buffers[10,17]. In the light of these findings, further experimentation would be advisable in order to establish whether amino acids or related compounds other than tryptophan are liberated in a greater amount under the action of sodium dodecylsulphate prior to sulphosalicylic acid deproteinization.

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