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Determination of plasma amino acids in small samples with the use of Dansyl-chloride (*).

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Summary. — A radiochemical method based on dansylation of plasma samples with Dansyl-chloride and thin layer chromatography on polyamide sheets is presented for the quantitative individual determination of plasma amino acids in very small blood samples. The results are first corrected with a norvaline internal standard and secondly with specific recovery factors for each amino acid. The results agree closely with other methods and with already published plasma normal amino acids values in healthy adult rats.

INTRODUCTION.

The use of 1-dimethylamino-naphtalen-5-sulphonyl chloride (Dansyl-chloride) [1] has been widely used as a probe of the amino terminal groups of amino acids, peptides and proteins [2]. This ability, united to the strength of the Dansyl-amino acid bond and to the characteristic fluorescence of these compounds [3] has made possible the use of this compound to identify and quantify the different amino acidic compounds present in biological fluids [4, 5, 6, 7]. The results attained with these procedures are fully comparable to other methods, namely the automatic chromatographic analyzer methods [8].

There is a considerable number of variation in the available methodology for the determination of amino acids using the Dansyl-chloride method, mainly with different reaction conditions and using different techniques, as paper electrophoresis [9] or more commonly thin layer chromatography [7, 8, 10] on silicagel or polyamide plates. There is also considerable variation on the method of quantification of the spots, being the more commonly used the direct measurement of fluorescence of the spots in the plate after its elution in an adequate solvent [10], the labelling with radioactive Dansyl-chloride and ulterior determination of the radioactivity in the spots [8] or densitometry after autoradiography [11].

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Internal standards of abnormal amino acids (norleucine or norvaline) or of [^{14}C]-labelled amino acids [8] have been used to make proper corrections of the results.

In this paper it has been intented to show a simplified and speedy procedure for handling a considerable number of samples in connection with an adequate deproteinization method already published [12]. It contains also adequate corrections for the effectiveness of the dansylation procedure and for the different affinity of the amino acids with the Dansyl moiety. This procedure is intended to be used for the quantitative determination of all amino acids present in the very small amounts of plasma available in our sequenced experiments with rats and mice, without affecting too much the blood volume of the animal.

EXPERIMENTAL. — MATERIALS.

Plasma was obtained from heparinized blood of healthy Wistar rats. Samples were stored at — 30°C until deproteinization.

All amino acids, Dansyl-amino acids and Dansyl-chloride were purchased from Sigma. Tritiated Dansyl-chloride (19 Ci/mM) and labelled amino acids were purchased from Amersham-Searle. *L*-[^{14}C]-Ala (UL), 10 mCi/mM ; *L*-[^{14}C]-Asn (UL), 100 mCi/mM ; *L*-[^{14}C]-Asp (UL), 208 mCi/mM ; *DL*-[3-^{14}C]-Cys, 25 mCi/mM ; *L*-[^{14}C]-Arg (UL), 150 mCi/mM ; *L*-[^{14}C]-Glu (UL), 249 mCi/mM ; [^{14}C]-Gly (UL), 50 mCi/mM ; *L*-(cycle-[^{14}C]-His, 57.8 mCi/mM ; *L*-[^{14}C]-Leu (UL), 4.63 mCi/mM ;

L-(methyl-[^{14}C])-Met, 25 mCi/mM ; L-[^{14}C]-Phe (UL), 522 mCi/mM ; L-[^{14}C]-Ser (UL), 75 mCi/mM ; L-[^{14}C]-Thr (UL), 100 mCi/mM ; L-[^{14}C]-Lys-HCl (UL), 150 mCi/mM ; L-[^{14}C]-Tyr (UL), 225 mCi/mM and L-[^{14}C]-Val (UL), 125 mCi/mM.

The chromatographies were run on plastic backed polyamide sheets (Eastman Chromagram Sheet, type K 541 V). The eluents and all other reagents used were analytical quality. Radioactivity countings were done in a Nuclear Chicago Isocap 300 scintillation counter fitted with external standard device. The efficiency curves for this apparatus were calculated with standards made with tritiated toluene from Amersham-Searle. All calculations and programs were run in a Compucorp Statiscian desk computer.

mantained 5 minutes at — 70°C and then lyophilized until dryness. The residue was dissolved in 0.005 ml of 0.1 M triethylamine and 0.005 ml of Dansyl-Chloride solution (5 mg/ml in acetone containing 0.2 µCi/ml of tritiated Dansyl-chloride) and incubated in stoppered tubes at 45°C during 10 minutes. The tubes were again freezed and lyophilized to complete dryness. The residue was treated with 0.025 ml of 6 N ClH containing 75 mM 2-mercaptoethanol in vials mantained into a gas-proof container with the same solution in the bottom. The container was then placed in an oven for 60 minutes at 105°C [9] and the hydrolysates were again dried out in the lyophilizer. The mixture of Dansyl-amino acids was then dissolved gently in 0.005 ml of 2 per cent formic acid in water and aliquots of 0.0005 ml were applied to

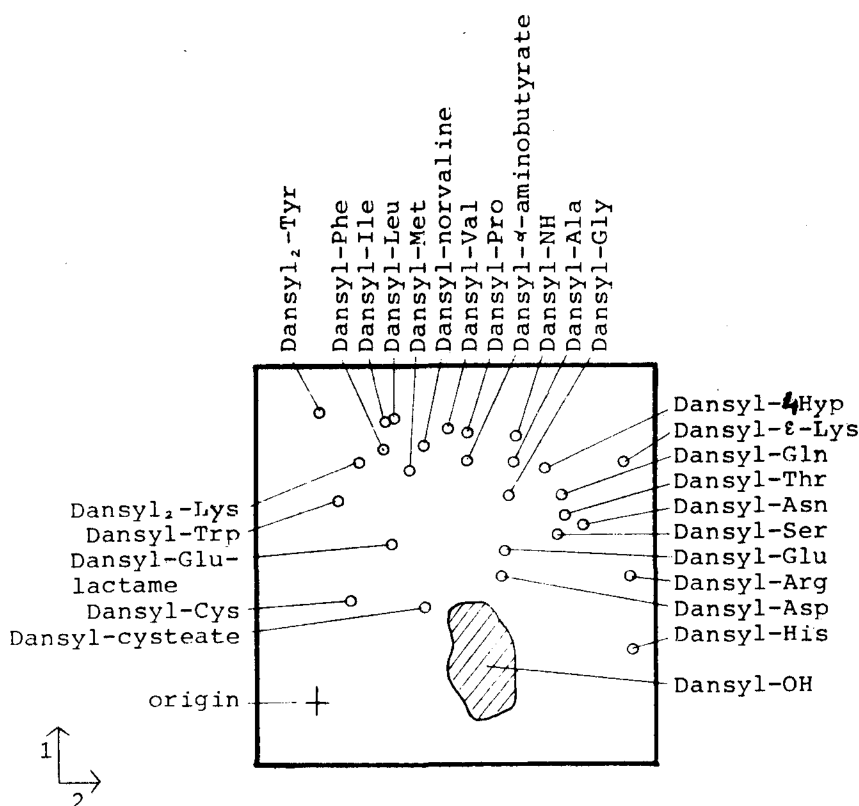

FIG. 1. — *Identification on a poliamide plate of the composite Rf values for the different Dansyl-amino acids studied.*

System 1 : *n*-butyl acetate/methanol/acetic acid (30:20:1). System 2 : 2 per cent formic acid in water.

METHOD AND DISCUSSION.

Dansylation procedure and Chromatography.

Plasma samples of up to 0.010 ml were deproteinized with acetone in small-bore capillary tubes [12]. The acetone containing supernantants were

the plates for bidimensional thin layer chromatography. The plates were cut at 65 × 65 mm and the eluents used were *n*-butyl-acetate/methanol/acetic acid (30:20:1 by volume) for the first run, and 2 per cent formic acid in water for the second (transversally with respect to the first one). The

samples were applied to the plates and these were developed in dim bulb light in order to prevent the action of the light upon the Dansyl-amino acids formed [13].

The developed chromatograms were visualized under 254 nm UV light and the spots outlined in pencil, numbered and cut with scissors. The individual fragments were dissolved in 0.250 ml of 80 : 20 ethanol/water (by volume) in a scintillation vial, and then 8 ml of a PPO/POPOP containing toluene/triton X-100 scintillation cocktail [14] were added for counting.

After developing the first plates it was found that there was a considerable amount of contamination in the plates, as ascertained by the UV inspection and by the radioactivity in the blank spots. The source of the contamination was tracked partially to the bidistilled water and to the sulfochromic acid-resistant contaminants found in the glassware. That made necessary to further deionize the water used and to clean all glassware with a sodium hydroxyde/nitric acid routine similar to the one described by Lowry and Passoneau [15]. In this way we obtained very good background plates with almost no contamination. Actually it was found unnecesary to wash the plates with redistilled chloroform [10]. In spite of its low radioactivity, the blank plates were cut in a way similar to the one indicated for the samples and the individual countings obtained were used as backgrounds for each spot.

Spot identification and recoveries.

The identification of the different spots belonging to the Dansyl-amino acids was accomplished in a triple way. First the plates were developed with individual known Dansyl-amino acids in order to obtain their composite Rf values (figure 1) and to localize their position on the developed plates. Second, these spots were identified by dansylation of amino acid mixtures and checking the situation of the spots. Third, plasma samples were supplemented with small amounts of labelled individual amino acids and their position checked by countings of the plates's fragments. In this way the spots indicated in figure 2, were identified corresponding to $Dansyl_2$-Tyr, Dansyl-Leu + Dan-

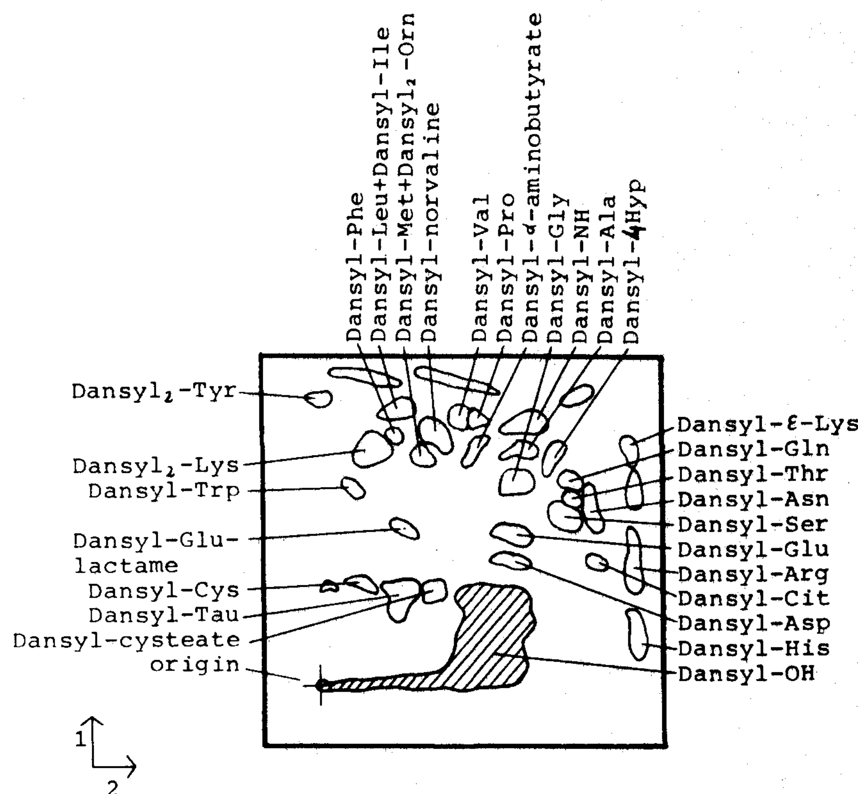

FIG. 2. — *Aspect of a typic dansylated plasma supernatant, showing the identified spots for the different Dansyl-amino acids.*

The elution systems used are the same as in figure 1.

syl-Ile, Dansyl-Val, Dansyl-NH_2, Dansyl-α-aminobutyrate, Dansyl-γ-aminobutyrate, Dansyl-Di-Lys, Dansyl-Met + Dansyl-Orn, Dansyl-Pro, Dansyl-Ala, Dansyl-4Hyp, Dansyl-ε-Lys, Dansyl-Gln, Dansyl-Gly, Dansyl-Trp, Dansyl-Asn, Dansyl-Ser, Dansyl-Glu-lactame, Dansyl-Glu, Dansyl-Arg, Dansyl-Asp, Dansyl-taurine, Dansyl-Cys, Dansyl$_2$-dicysteine, Dansyl-cysteate and Dansyl-OH. It was also determined the situation of Dansyl-norvaline that later was used as internal standard.

Labelled amino acids were added to plasma and the recoveries in the spots were calculated as a percent of the initial radioactivity present in the plasma sample. The results obtained showed acceptable recoveries for added amino acids, with a mean ± s.e.m. of 25.81 ± 2.89 for 16 different amino acids (5 determinations each). The dispersion of the results was, however, considerable, due to day to day variations and differences from one amino acid to another. As a way to cope with this lack of repetitivity we used norvaline as internal standard, taking advantadge of its absence in normal plasma and its clear isolation in our system. To each plasma sample it was added a fixed known amount of norvaline prior to its deproteinization. The results we corrected by the recovery of the norvaline, giving very good repetibility. Following this procedure, we studied the recoveries of individual amino acids. Individual amino acids and mixtures of different amino acids were dansylated as indicated above, and the radioactivity in the spots corrected with norvaline. The results are summarized in figure 3, in which there are shown the recoveries of individual amino acids versus the amounts added. The recoveries change linearly with the amount of each amino acid in the range used. The recoveries of different amounts of the same amino acid added to plasma give the same straight lines. From the slopes of these correlation lines the correction factors of individual amino acids were calculated. These factors are shown in table I and may be used as indexes of the affinity of this dansylation and extraction procedure for each amino acid with regard to norvaline.

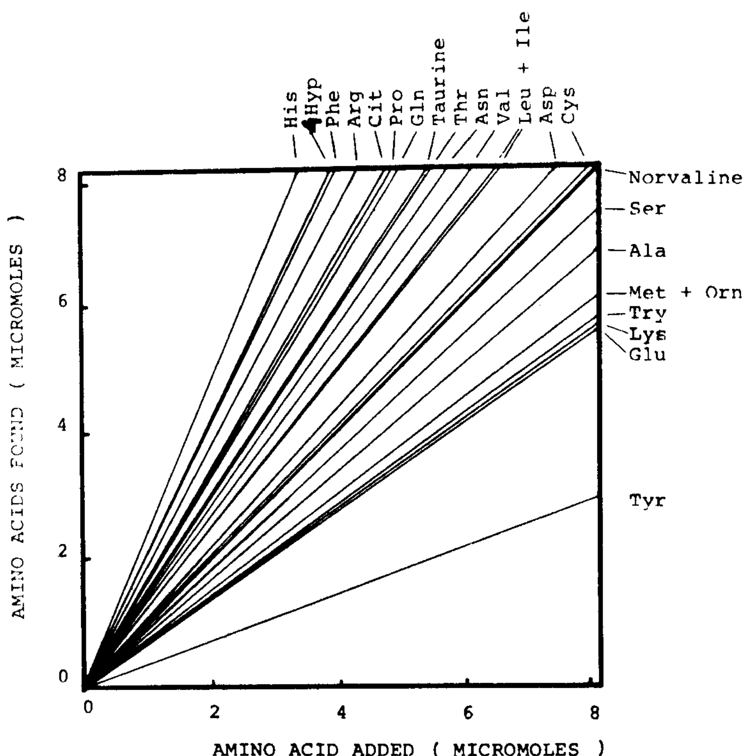

FIG. 3. — *Recovery lines of added amino acids to plasma samples with regard to the norvaline recuperation curve.*

The slopes of these curves are the correction factors shown in table I.

In order to process the data automatically we devised a simple desk computer program that takes the cpm in the norvaline spot and their counting external standard ratio and calculates

(after substracting its individual background) the dpm corresponding to the nanomoles added to the plasma sample. The cpm of the amino acid spots from each sample minus the individual back-

TABLE I.

Correction factors for the different individual amino acids according with the norvaline recoverie.

Amino acid	Correction factor
Glu	0.806
Gln	1.062
Ala	1.148
Lys	1.387
Taurine	0.667
Pro	0.589
Ser	1.063
Leu + Ile	0.785
Trp	1.353
Gly	0.624
Tyr	1.030
Val	0.726
Thr	0.926
Arg	0.438
Asp	0.896
Asn	0.742
Met + Ornithine	1.294
Cys + half-cystine	0.963
Citrulline	0.577
His	0.468
Phe	0 489
4Hyp	0.437

The values given are corrected for actual recuperation in the chromatographic plates after adjusting the general recovery by the means of a norvaline internal standard.

grounds are converted to dpm according to their external standard ratios. These dpm are converted to nanomoles in the original sample of blood using the norvaline correction factor and finally each value is converted into corrected micromoles per liter after multiplying for the corresponding factor already outlined in table I.

Under the conditions outlined, however, it was found that some part of the glutamine present in the samples was deamidated and determined as glutamate. Using pure amino acid solutions it was possible to determine their adequate correction factors and almost negligible overlapping was observed. The same is true for Asp and Asn. Unfortunately, with the use of rat plasma, higher amounts of glutamate formed from Gln were observed. This problem was partly due to the storage conditions of our samples and to the deamidation taking place in our experimental layout. To obviate this posible artifact we give here the composite values for Glu + Gln (Glx) and for Asp + Asn (Asx) in the table II, regardless of the ability of the method for differentiating both sets of amino acids.

Values of plasma amino acids in normal rats.

The results found for normal rat plasma (table II) are in close accordance with the data found in the current literature for this same animal [16 to 27]. Total amino acids in plasma gave good reproducibility. It is interesting to point out that in quantitative terms the most important amino acids found were the gluconeogenetic ones (Glx, Ala, Ser, Pro) [17, 21, 23, 24, 25] together with the branched chain ones (Leu, Ile and Val) [16, 18, 26, 27]. It was found that the amino acid proline was present in considerable amounts, comparable to the ones found by several authors [19, 21, 22] but higher [23, 26, 27] or lower [17] than those found recently by others. This amino acid, together with the 4Hyp is perfectly quantified with this method contrarily to the automatic ninhidrin-based amino acids analyzers, that fail to detect these materials.

TABLE II.

Concentration in micromoles/liter of the different amino acids found in the plasma of healthy adult Wistar rats.

Amino acid	Concentration
Glx (Glu + Gln)	873.35 ± 42.11 *
Ala	721.63 ± 25.90
Lys	512.80 ± 29.74
Taurine	421.21 ± 41.60
Pro	395.97 ± 22.53
Ser	356.40 ± 14.71
Leu + Ile	345.44 ± 17.20
Trp	278.11 ± 14.79
Gly	272.58 ± 13.44
Tyr	193.27 ± 16.23
Val	191.94 ± 10.03
Thr	181.37 ± 6.67
Arg	176.84 ± 9.42
Asx (Asp + Asn)	159.37 ± 9 67
Met + Ornithine	134.98 ± 10.01
Cys + half-cystine	110 43 ± 6.88
Citrulline	85.42 ± 5.66
His	74.49 ± 5.11
Phe	63.19 ± 2.22
4Hyp	54.58 ± 2.53
Total....	5605.86 + 98.36

(*) All data are mean ± s.e.m. of 20 determinations.

Sulfur amino acids and taurine are well differenciated with this method, obtaining values comparable with the ones described in the literature [16, 23, 26] but somewhat higher than in other papers [17, 20, 22, 25] ; our maximum is in form of taurine as in elsewhere [16]. The other amino acids, mainly essential ones, present values very much close to the ones described with the exception of tyrosine, that is constantly found in a level somewhat higher and Trp, whose [28] values are more in accordance with the « total » Trp in human plasma than with the values indicated in the literature for rats. Probably it has some relationship with the method of deproteinization used, as the one applied here does not leave any Trp bound to the protein [12] while other deproteinization methods currently used do not free all Trp present in the plasma. The values for Lys are high compared with some described results [16, 21, 23, 25], but equal [18, 22, 24, 26, 27] or lower [17, 19] than others. Anyway there is a definite trend in our results on this last group of amino acids of being somewhat high in comparison with those of other authors. We consider that this can be due to the high rich-protein content of the diet eaten by our experimental animals.

The total figure for amino acids found in this paper, 5605.86 ± 98.36 micromoles/liter is in very close accordance with the results obtained for total amino acids with a fluorometric technique using fluorescamine [29, 30] or with a complexometric method (correcting for the imino acids and taurine) [31] using the same rat plasma samples. In both cases the differences were lower than a ± 3.2 per cent. The total amino acids figure indicated is in accordance with published data [24] and is higher than others [18, 22, 25], due to the probable uncompleteness of the latter, as no data for Gln or Taurine are given.

It is concluded, then, that this method constitutes a considerable improvement in the current methods for determination of individual amino acids in very minute samples using the Dansylchloride technique rutinarily, circumventing most of the problems that this particular methodology poses on the adequate expression of the results.

RÉSUMÉ.

On présente une méthode radiochimique pour la détermination quantitative des acides aminés du plasma, avec des échantillons très petits du sang. Cette méthode est fondée sur la dansylation des échantillons avec le chlorure de dansyl et chromatographie en couche mince sur plaques de polyamide.

Les résultats doivent être corrigés avec un standard interne de norvaline et à l'aide des facteurs de récupération spécifiques pour chaque acide aminé. Les résultats sont en accord avec ceux obtenus par d'autres méthodes et avec les taux normaux des acides aminés du plasma chez des rats adultes.

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