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Note

Separation and identification of dansylated human serum and urinary amino acids by two-dimensional thin-layer chromatography

Application to aminoacidopathies

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Various authors have utilized the sensitivity of fluorescent labelling in the qualitative and quantitative analysis of the dansylated derivatives of amino acids and peptides [1, 2]. The isolation of these derivatives polyamide thin-layer chromatography was first described in 1967 by Woods and Wang [3], and has been adapted for micro determination [4]. Later, the optimal conditions for dansylation and its application to the separation of known amino acid mixtures or biological samples were reported. This technique, however, has never been used for analysis of human serum and urinary amino acids or for screening for aminoacidopathy.

The method described differs from classical chromatographic techniques on cellulose or silica gel, both mono- and two-dimensional, by its speed (migration length 3.5 cm), its excellent resolution, and its sensitivity (average amount deposited 50 picomoles). In addition, it can be applied to non-deionised urine samples and to non-deproteinised ultrafiltrated serum.

MATERIALS AND METHODS

Reagents

Dansyl chloride and free amino acids were obtained from Sigma (St. Louis,

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MO, U.S.A.); the other chemicals were from Merck (Darmstadt, G.F.R.). The micropolyamide plates (F 1700, 15 × 15 cm) were from Schleicher and Schüll (Dassel, G.F.R.). Micro tanks (4.5 × 3 × 9 cm) were used for migration.

The amino acid reference solutions (10^{-1} *M* in 0.1 *N* HCl) were stored at −20°C and diluted for dansylation to 10^{-3} *M* with 0.05 *M* $NaHCO_3$ solution. The mobile phases used are (I) formic acid—water (1.5:98.5), (II) benzene—acetic acid (4.5:1).

The 0.05 *M* $NaHCO_3$ solutions and the solvents can be stored up to one week, at 4°C, in airtight containers.

Sample preparation

Daily urines are collected with a preservative (chloroform—water, 4:1000, v/v). A choice had to be made between chloroform and other preservatives such as organo-mercuric derivatives, e.g. 1% merseptyl®. This latter product gives a dansylated derivative which does not interfere with other spots of the chromatogram. On the other hand, sodium azide should be ruled out because it quenches fluorescence. The serum is dialyzed by ultrafiltration (1 h, 4 bars) through a collodion membrane filter (Sartorius). Urine and serum ultrafiltrates can be stored at −20°C; they are adjusted to pH 8.5 with $NaHCO_3$ for the dansylation reaction. Biological samples and standards are dansylated by mixing (v/v) with fresh dansyl chloride solution (3.7 m*M* in acetone) and incubating for 1 h at 37°C. The dansyl amino acids can be stored for up to 1 month at −20°C in the dark.

Chromatography

The volume of dansylated standards applied is 0.1 µl, which corresponds to approximately 50 picomoles per amino acid (detection limit of dansylated glycine is 1.25 picomoles).

For urine the volume deposited, *X* (µl), is calculated on the basis of the daily urine volume, *V* (liters), corrected for the body surface area, *S* (m^2), of the child (adult surface = 1.73 m^2). The formula applied is $0.1 \times V \times (1.73/S)$. *S* is found from Dubois tables [5] based on the height (cm) and weight (kg) of the child. This formula takes into consideration diuresis, body surface and methodological sensitivity. However, it is limited, and does not take into account many parameters such as age, sex, pregnancy, inaccuracy of child samples, that are liable to modify, qualitatively and quantitatively, the aminoaciduria.

Some authors propose basing the volume on the creatinine content. This method, while valid for adults, is less applicable to infants and young children because of large individual and daily variations.

We advise, for the pathological interpretation, that a second chromatogram be prepared using a sample taken from a normal patient belonging to a similar age and population group. Thus, in the case of an adult male with a daily urine volume of 1 liter and a standard 1.73 m^2 body surface, according to the values obtained by Lewis et al. [6], the maximum difference between applied quantities will not exceed 100 picomoles for glycine and 2.5 picomoles for α-aminoadipic acid.

For plasma, 0.1 µl of dansylated ultrafiltrate is sufficient to take into account the extreme physiological values of an adult male. As in aminoaciduria,

the evaluation is based on the comparison of a chromatogram from a normal patient of similar age.

The aliquots are applied with a 1-μl microsyringe with an unbevelled needle in 0.05-μl fractions (spot 0.5 mm in diameter) at 5 mm from each contiguous side of a previously cut micropolyamide plate (4.2 × 4.2 cm). Plates carefully handled with gloves are introduced into perfectly flat presaturated tanks. The solvents are removed following each analysis.

After allowing free migration for 5 min in solvent system I, the plates are dried with hot air and then cooled to room temperature. They are then placed in solvent system II and allowed to migrate for 5 min in a direction perpendicular to the first.

Detection and interpretation

The yellow fluorescent spots are identified under light at 254 nm (Camag TL 900U). Dansyl sulfonic acid exhibits a blue fluorescence. The migration distances of the dansyl amino acids in systems I and II are measured in comparison with a dansyl glycine internal standard.

Interpretation is carried out by comparison with control chromatograms. The stability of the fluorescence in the absence of light allows easy storage of chromatograms and comparison with further sequential biological samples. The entire analysis requires 3 h when performed by an experienced operator.

RESULTS AND DISCUSSION

Thirty-nine amino acids representative of normal or pathological samples have been studied individually, pooled in structural and functional groups A, B, C, D, E, and F (Fig. 1), and in a global pool (groups A + B + C + D + E + F, Fig. 2).

Dual identification of the different spots of the dansyl amino acids was possible. Firstly the plates were developed individually for each known dansylated amino acid in order to localize its position. Secondly, the spots were identified after dansylation of the amino acid mixtures (A, B, C, D, E, and F from Fig. 1, or the global pool A + B + C + D + E + F from Fig. 2), and by checking the location of each spot.

Certain amino acids (tyrosine, lysine, cysteine, etc.) contain two reactive functions and can produce two or three spots (two monodansylated derivatives and one bisdansylated derivative). Practically, the migration distances are measured from the most fluorescent spot identified (generally the bidansylated derivative) in a separate experiment with pure commercial standards (Sigma, Cat. No. DAN L 23).

The relative migration distances, determined as the value R_x, are measured after the migration of the pooled amino acids and in comparison with a glycine internal standard (Fig. 1). Table I shows the R_x values obtained in solvent systems I and II, arranged in increasing order. The procedure, which consists of supplementing known individual amino acids, is suitable for identifying any amino acid present at abnormal levels. Fig. 2 indicates the relative positions of each amino acid studied and demonstrates the sharpness of the separation. Nevertheless, some amino acid associations remain unresolved, especially

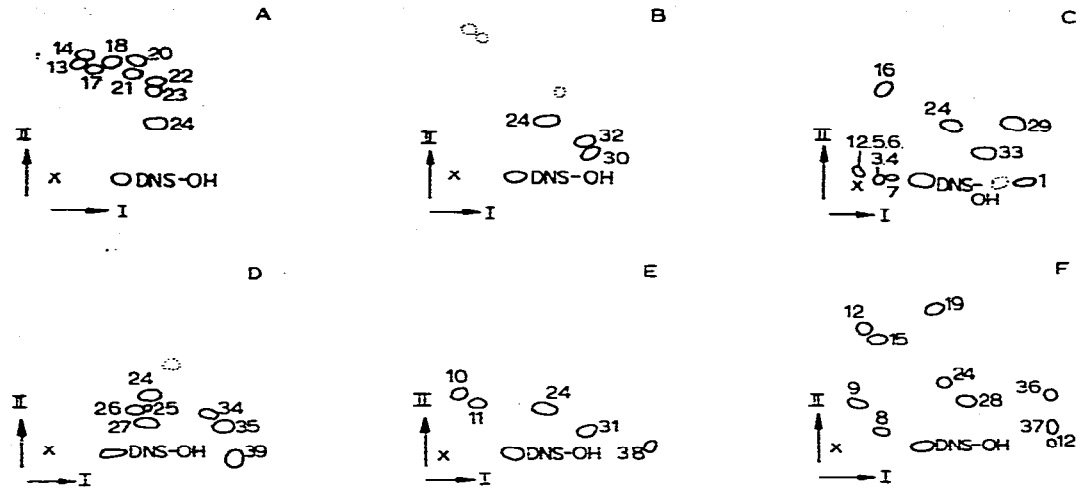

Fig. 1. Two-dimensional chromatography in solvent systems I and II of known dansylated amino acids pooled in structural and functional groups A, B, C, D, E and F. Identification was with glycine as internal standard. DNS-OH = dansyl sulfonic acid. (A) 13 = leucine, 14 = isoleucine, 17 = norvaline, 18 = valine, 20 = β-aminoisobutyric acid, 21 = GABA (γ-aminoisobutyric acid), 22 = β-alanine, 23 = alanine, 24 = glycine. (B) 30 = serine, 32 = threonine, 24 = glycine. (C) 1 = cystine, 2 = cysteine, 3 = taurine, 4 = taurocholic acid, 5 = homocysteine, 6 = homocystine, 7 = cysteic acid, 16 = methionine, 29 = L-methionine sulfoxide, 33 = DL-methionine sulfone, 24 = glycine. (D) 26 = glutamic acid, 27 = aspartic acid, 34 = glutamine, 35 = asparagine, 25 = α-aminoadipic acid, 39 = arginosuccinic acid, 24 = glycine. (E) 10 = lysine, 11 = ornithine, 31 = citrulline, 38 = arginine, 24 = glycine. (F) 8 = tyrosine, 9 = tryptophan, 12 = histidine, 15 = phenylalanine, 19 = proline, 28 = hydroxyproline, 36 = 3-methylhistidine, 37 = 1-methylhistidine, 24 = glycine.

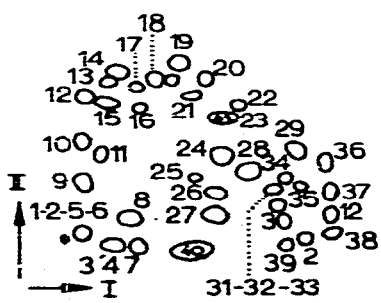

Fig. 2. Two-dimensional chromatography of a global pool of 39 known dansylated amino acids (groups A + B + C + D + E + F from Fig. 1). Identification was with glycine as internal standard. 1 = cystine (bis-dansyl), 2 = cysteine, 3 = taurine, 4 = taurocholic acid, 5 = homocysteine, 6 = homocystine, 7 = cysteic acid, 8 = tyrosine (bis-dansyl), 9 = tryptophan, 10 = lysine (bis-dansyl), 11 = ornithine (bis-dansyl), 12 = histidine (bis-dansyl), 13 = leucine, 14 = isoleucine, 15 = phenylalanine, 16 = methionine, 17 = norvaline, 18 = valine, 19 = proline, 20 = β-aminoisobutyric acid, 21 = GABA (γ-aminoisobutyric acid), 22 = β-alanine, 23 = alanine, 24 = glycine, 25 = α-aminoadipic acid, 26 = glutamic acid, 27 = aspartic acid, 28 = hydroxyproline, 29 = L-methionine sulfoxide, 30 = serine, 31 = citrulline, 32 = threonine, 33 = DL-methionine sulfone, 34 = glutamine, 35 = asparagine, 36 = 3-methylhistidine, 37 = 1-methylhistidine, 38 = arginine, 39 = arginosuccinic acid, 40 = dansyl sulfonic acid, 41 = dansylamine.

TABLE I

R_x VALUES OF DANSYLATED AMINO ACIDS SEPARATED BY TWO-DIMENSIONAL CHROMATOGRAPHY ON MICROPOLYAMIDE PLATES

	R_{x_1}*		R_{x_2}**
Tyrosine (bis-dansyl)	0	Arginosuccinic acid	0
Cysteine (bis-dansyl)	0.11	α- or β-dansylcysteine	0
Cystine (bis-dansyl)	0.11	Taurine	0
Homocysteine	0.11	Taurocholic acid	0
Homocystine	0.11	Cysteic acid	0
Tryptophan	0.13	α-Amino dansylhistidine	0.05
Lysine (bis-dansyl)	0.13	Cysteine (bis-dansyl)	0.17
Histidine (bis-dansyl)	0.19	Cystine	0.17
Leucine	0.25	Homocysteine	0.17
Isoleucine	0.29	Homocystine	0.17
Taurine	0.30	Arginine	0.24
Taurocholic acid	0.30	Tyrosine (O-dansyltyrosine)	0.27
Ornithine	0.32	1-methylhistidine	0.30
Cysteic acid	0.38	Aspartic acid	0.32
Phenylalanine	0.38	Asparagine	0.42
Tyrosine (O-dansyltyrosine)	0.39	Serine	0.46
Norvaline	0.42	Glutamic acid	0.53
Methionine	0.49	Glutamine	0.58
Valine	0.56	Citrulline	0.59
γ-Aminoisobutyric acid	0.75	DL-Methionine sulfone	0.66
DL-β-Aminoisobutyric acid	0.80	Threonine	0.67
Proline	0.88	Tryptophan	0.68
Alanine	0.92	Hydroxyproline	0.73
Glutamic acid	0.92	DL-α-Aminoadipic acid	0.80
Aspartic acid	0.93	3-Methylhistidine	0.80
β-Alanine	1.00	Glycine	1.00
Glycine	1.00	L-Methionine sulfoxide	1.08
DL-α-Aminoadipic acid	1.00	Ornithine	1.12
Hydroxyproline	1.19	Lysine (bis-dansyl)	1.32
DL-Methionine sulfone	1.33	Methionine	1.54
Threonine	1.35	Alanine	1.55
Serine	1.38	Phenylalanine	1.64
Citrulline	1.45	β-Alanine	1.70
L-Methionine sulfoxide	1.50	Tyrosine (bis-dansyl)	1.75
Glutamine	1.57	γ-Aminoisobutyric acid	1.82
Asparagine	1.72	Histidine (bis-dansyl)	1.86
Arginosuccinic acid	1.80	Norvaline	1.91
α- or β-dansyl cysteine	2.00	Leucine	1.95
1-Methylhistidine	2.02	DL-β-Aminoisobutyric acid	2.00
α-Amino dansylhistidine	2.02	Valine	2.00
3-Methylhistidine	2.02	Isoleucine	2.09
Arginine	2.08	Proline	2.09

*$R_{x_1} = \frac{\text{migration distance of dansyl amino acid}}{\text{migration distance of dansyl glycine}}$ in solvent system I (formic acid—water, 1.5 : 98.5.

**$R_{x_2} = \frac{\text{migration distance of dansyl amino acid}}{\text{migration distance of dansyl glycine}}$ in solvent system II (benzene—acetic acid, 4.5 : 1).

cysteine/cystine/homocysteine/homocystine (perhaps due to the breaking of the covalent disulfide bond when dansylated), taurine/taurocholic acid and citrulline/threonine. Interference by excess dansylsulfonic acid, with the fluorescence of cysteic acid, previously described by Lee and Safille [1], was not observed under our conditions.

The dansylamine by-product of the dansylation reaction [7] shows a yellow fluorescence at the same place as dansyl alanine. The two derivatives can be separated by a third migration, in the second dimension, with Na_2PO_4 (0.05 *M*)—ethanol (3:1) [4]. In addition, this procedure increases the resolution of the dansyl arginine/bisdansyl lysine couple.

CLINICAL APPLICATIONS

Fig. 3a shows chromatograms for serum and urine of a child with leucinosis. The serum chromatogram points out an obvious increase in leucine, isoleucine and valine. In the same way, the quality of serum chromatography of other aminoacidopathies, notably for a case of citrulinemia (Fig. 3b) and a case of phenylketonuria (Fig. 3c), emphasises the interest of analysis of a serum milieu whose composition is more constant than that of urine.

In conclusion, the major advantage of the proposed method is its qualitative evaluation of general or a particular aminoacidopathy.

In comparison with classical thin-layer chromatographic (TLC) separation on cellulose or silica gel, the proposed identification of human aminoacidopathies

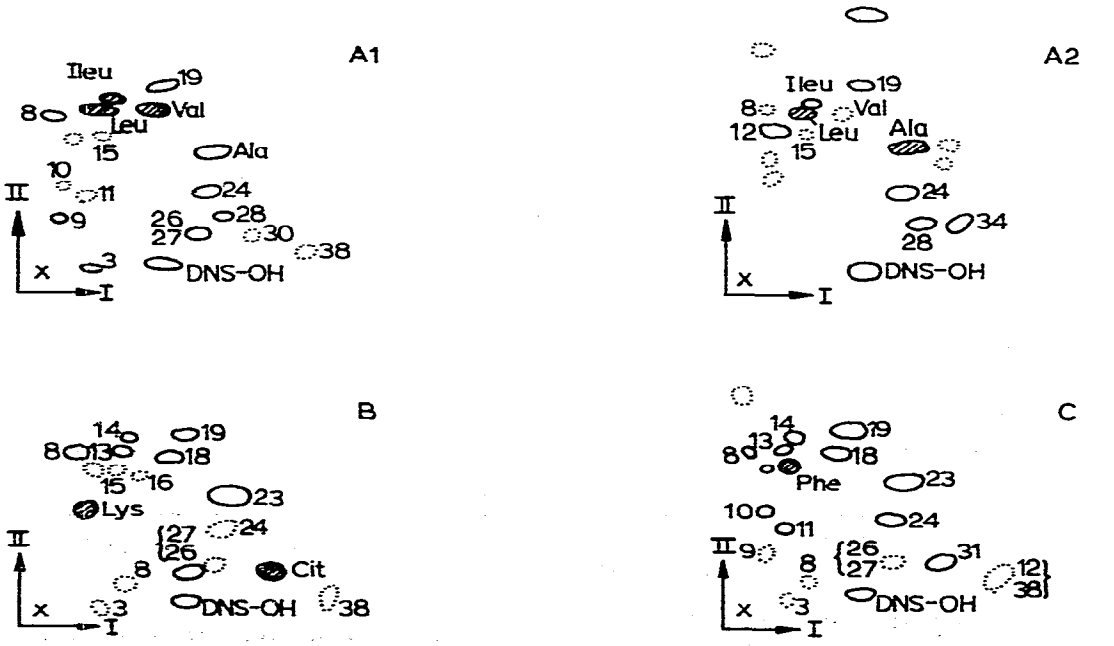

Fig. 3. Chromatograms of serum and urine from different children with leucinosis, citrullinemia and phenylketonuria: (A) leucinosis (A_1 = serum, A_2 = urine); (B) citrullinemia (serum); (C) phenylketonuria (serum).

by TLC on polyamide is faster, with greatly improved definition (no tails) and does not require deionised samples. The results justify analysis using serum, which is of a more constant composition than urine, and which shows more clearly any pathology, giving a more accurate diagnosis, especially in the case of aminoacidopathies associated with secondary nephropathy.

Quantitatively, the separated dansylated amino acids can be eluted and assayed following the technique described by Airhart et al. [8]. The sensitivity (about 1 picomole) and the rapidity of this method might provide a valuable alternative to standard autoanalyzer determination.

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