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DETERMINATION OF 27 DANSYL AMINO ACID DERIVATIVES IN BIOLOGICAL FLUIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The concentrations of free amino acids in plasma and in ascitic liquid of mice with Ehrlich ascitic tumours were determined by reversed-phase high-performance liquid chromatography using pre-column derivatization with Dns chloride and UV detection at 254 nm. Sample preparation is simple, and the Dns derivatives are stable. Complete separation of 27 amino acids, including proline and cysteine, was achieved in 70 min with detection limits of less than 25 pmol. There was no interference from Dns-Cl, Dns-OH and Dns-NH_2. Retention time reproducibility was better than 1%. The described method enables a rapid, economical and reproducible quantification of free amino acids in biological fluids.

INTRODUCTION

A number of reports on the analysis of amino acids have appeared in the last few years. High-performance liquid chromatography (HPLC) appears to be a valid alternative to classical ion-exchange chromatography with subsequent post-column ninhydrin derivatization [1]. The principal disadvantages of this technique compared with the reversed-phase mode are well known. Pre-column derivatization of amino acids is performed using phenylthiohydantoin (PTH) [2], 1-dimethylaminonaphthalene-5-sulphonyl (dansyl, Dns) [3, 4] or *o*-phthalaldehyde (OPA) [5, 6]. The dansylation was selected for the present study because the quantitative preparation of the amino acid derivatives is easy and they are stable [3, 7]. The use of Dns derivatives also avoids the loss of cysteine, proline and hydroxyproline, which cannot be detected by the OPA method [8]. Separations of Dns-amino acids by electrophoresis [9], thin-layer chromatography [10, 11] and HPLC [3, 12—14] have been reported previously. However, most of these studies have dealt with the separa-

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tions of standard amino acid solutions and there are few data concerning sample preparation for quantitative HPLC analysis of free amino acids in biological fluids [15—18]. The method proposed in the present report enables the determination of 27 amino acids in plasma and ascitic fluids of tumour-bearing mice within 70 min. It uses dansylation prior to reversed-phase separation and UV detection.

EXPERIMENTAL

Apparatus

A Spectra-Physics (San José, CA, U.S.A.) HPLC system was used, equipped with a Model SP 8700 solvent delivery system, a Model 7125 Rheodyne injection valve with a 10-μl sample loop, a Model SP 8700 pump and a Model SP 8300 variable-wavelength detector (254 nm; 10-mm flow-cell, 10 μl) with an electronic control unit. Continuous on-line quantification of chromatographic peaks was effected using a Model SP 4100 computing integrator. Separation of Dns derivatives was carried out on a 5-μm Supelcosil LC-18 reversed-phase column (150 × 4.6 mm I.D., Supelco, Bellefonte, PA, U.S.A.). The analytical column was protected by a MPLC New Guard holder containing a 7-μm disposable New Guard RP-18 cartridge (15 × 3.2 mm I.D., Brownlee Labs., Santa Clara, CA, U.S.A.). All chromatographic separations were performed at constant room temperature.

Reagents and chemicals

HPLC ultra-pure water generated by a Milli-RO 60 coupled to a Milli-Q water purification system (Millipore, U.S.A.) was used to prepare the aqueous solutions. HPLC-grade methanol, analytical-grade lithium carbonate, acetonitrile, acetic acid and synthesis-grade triethylamine were purchased from Merck (Darmstadt, F.R.G.). Dns-Cl, free amino acids and their Dns derivatives were from Sigma (St. Louis, MO, U.S.A.). Individual standard stock solutions (1 m*M*) of amino acids and Dns-amino acids were prepared in 40 m*M* lithium carbonate buffer (pH 9.5) adjusted with hydrochloric acid and stored at 4°C. A standard mixture containing all the amino acids, designed to simulate the physiological fluid, was made up. Solvents, standard solutions and samples were filtered before the analyses through 0.45-μm Durapore filters using a Swynny portafilter (Millipore, U.S.A.).

Animals and tumour samples

Two-month-old female albino Swiss mice, purchased from Panlab (Barcelona, Spain) were inoculated in the peritoneal cavity with $5 \cdot 10^6$ cells of a hyperdiploid Lettré strain of Ehrlich ascites tumour kindly supplied by Dr. T. Galeotti (Universitá Cattolica Sacro Cuore, Rome, Italy). The tumour cells were harvested about 7 days after inoculation, and the ascitic fluid was obtained by immediate centrifuging of the tumoral suspension at 2000 *g* for 5 min at room temperature. The animals received standard Panlab food with tap water ad libitum. They were kept at approximately 24°C with an 8 a.m.—8 p.m. light—dark schedule.

Preparation of biological samples

Blood samples were taken using heparinized Pasteur pipettes from an axillary incision made under ethylic anaesthesia. The blood was transferred into 1.5-ml Eppendorf tubes containing 10 μl of 50 mg/ml heparin in 0.15 *M* sodium chloride solution, and immediately centrifuged at 2000 *g* for 5 min. Plasma or ascitic fluid samples (100 μl) were deproteinized by adding four volumes of methanol, gently shaken, and allowed to stand for 10 min at 4°C, then centrifuged for 5 min at 11 600 *g*. The supernatant was collected and the protein precipitate was washed three times with 100 μl of a methanol—water mixture (4:1), and centrifuged at 11 600 *g* for 5 min. All the supernatants were pooled. The methanol was evaporated from the supernatants in an oven at 80°C to approximately 30—50 μl, adjusted to the original volume with water and stored at —30°C until analysis. To obtain a quantitative recovery of amino acids from the samples, it is very important not to evaporate the samples to dryness because the amino acid mixture cannot be redissolved. Furthermore, the protein precipitate must be washed several times. We observed that more than 50% of some free amino acids were trapped in the protein precipitate. The deproteinization efficiency was better than 99%. Protein was measured by the method of Lowry et al. [19] using bovine serum albumine as a standard.

Dansylation of amino acids

Dns derivatization was carried out according to the method of Tapuhi et al. [12], using Dns-Cl in acetonitrile (usually 5.5—20 m*M*) and a 40 m*M* lithium carbonate solution (pH 9.5) as a reaction buffer, for 1 h at room temperature. The molar excess of Dns-Cl for optimal dansylation should be 5—10 times the total amino acid content. The ratio of Dns-Cl solution to amino acid solution was 1:2. For biological samples, 100 μl of the deproteinized plasma or ascitic fluid were treated with 100 μl of reaction buffer and 100 μl of 20 m*M* Dns-Cl. After stirring, the mixture was allowed to stand for 1 h at room temperature. In all cases, the reaction vials were protected from light with aluminimum foil. Under these conditions, cystine, ornithine, lysine, histidine and tyrosine gave di-Dns derivatives.

TABLE I

CHROMATOGRAPHIC CONDITIONS FOR HPLC ANALYSIS OF Dns-AMINO ACID DERIVATIVES

Solvents: A, methanol; B, 0.6% acetic acid—0.008% triethylamine in water.

Duration (min)	Flow-rate (ml/min)	From ratio A:B	To ratio A:B	Gradient
0—20	1	30:70	30:70	—
20—25	1	30:70	40:60	Linear
25—50	1.5	40:60	50:50	Linear
50—55	2	50:50	50:50	—
55—65	2	50:50	65:35	Linear
65—70	2	65:35	75:25	Linear
70—75	2	75:25	75:25	—

Chromatographic procedure

Two mobile phases were used: (A) methanol and (B) 0.6% acetic acid with 0.008% triethylamine in water solution. The gradient program applied to the two helium-conditioned solvents is summarized in Table I. Amino acid concentrations were determined by external calibration method. The calibration mixtures were analysed in triplicate. The computer unit was programmed for baseline correction, valley-to-valley, to compensate for the baseline shifts caused by increases in non-polar solvents and of flow-rates towards the end of the chromatographic runs.

RESULTS AND DISCUSSION

Optimization of chromatographic separation

The aim of the present work was to devise and optimize a rapid, economical and reproducible method to quantify free amino acids in physiological samples. The work of our group is focused on the nitrogen movements between host and tumour in mice inoculated with Ehrlich ascitic tumour cells [20]. Consequently, it is fundamental to determine the essential and non-essential free amino acids in plasma and ascitic liquid to calculate the nitrogen fluxes.

Fig. 1 shows the chromatogram of a mixture of 27 standard Dns-amino acid derivatives. Under the conditions described in Table I, there was good resolution of all amino acids except for the phenylalanine—isoleucine pair. It is noteworthy that the cysteine and proline derivatives (amino acids undetected by the OPA method) were also resolved. Dns-NH_2 was clearly separated from the glutamine and serine peaks and, because of the short retention times of Dns-Cl and Dns-OH, they did not interfere with the amino acid deriv-

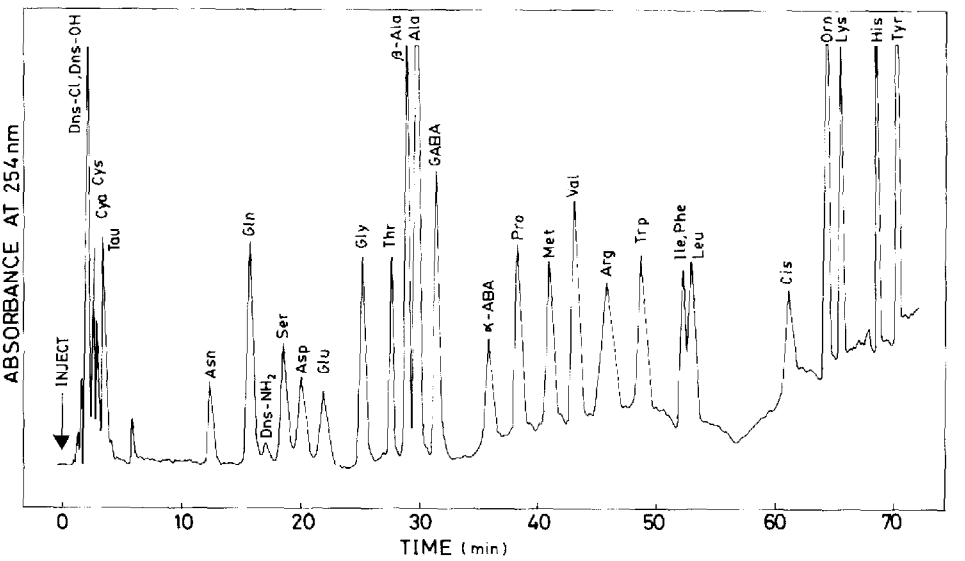

Fig. 1. Chromatogram of a standard mixture containing 27 Dns-amino acid derivatives. The concentrations of all amino acids were of the order of their plasma levels (15—75 nmol/ml). The elution program is described in Table I. Injection volume 10 µl. For experimental details, see Experimental section.

atives. This is in contrast with the chromatograms recently published by Martin et al. [21]. An initial, 20-min isocratic elution was required to resolve the most polar amino acids (Table I). The aspartate—glutamate pair and the threonine—glycine pair were clearly resolved in this way. However, other authors, using Dns derivatives, have reported that these two pairs either co-eluted or were poorly resolved [14, 21]. At 50 min after sample injection, the flow-rate was increased to 2 ml/min to elute the most hydrophobic compounds: the di-Dns derivatives of ornithine, lysine, histidine, cystine and tyrosine. The total separation time was 70 min. This time is shorter than the separation time reported previously by Oray et al. [14]. The method is thus suitable for the analysis of biological samples.

Quantification and reproducibility

The reproducibility of retention times of the 27 standard Dns-amino acids, and of the free amino acid derivatives occurring in plasma, was calculated from fifteen analyses. Except for glutamate, the coefficient of variation (C.V.) was always less than 1.0%, both for the standard and the biological samples. Great care must be taken to avoid changes of temperature and solvent composition; constant values of these two parameters are critical [14] to obtain adequate separation of the valine—arginine pair.

The reproducibility of the peak areas was also studied. The average C.V. for standard derivatives was better than 3.4%. Slightly higher C.V. values were found for plasma samples compared with the reference compounds. In samples of biological fluids, the largest values were found for asparagine and arginine; these variations may be explained by the low concentration of asparagine in plasma and by the broad shape of the arginine peak. The average recovery of amino acids added to the plasma was 99.7 ± 3.3% (Table II), showing the accuracy of the method.

All the amino acid derivatives tested showed linear responses in the concentration range of 50—750 pmol. Dns derivatives were stable for two weeks

TABLE II

RECOVERY OF AMINO ACID STANDARDS ADDED TO PLASMA

Amino acid	Amino acid in sample (nmol)			
	Plasma alone	Plasma + 2.50 nmol (actual)	Plasma + 2.50 nmol (theoretical)	Recovery (%)
Tau	10.25	13.15	12.75	103.14
Asn	1.29	3.77	3.79	99.47
Gln	12.51	14.86	15.01	99.00
Ser	4.14	6.62	6.64	99.70
Gly	9.71	12.76	12.21	104.50
Thr	4.55	7.19	7.05	101.98
Ala	7.81	10.23	10.31	99.22
Pro	4.36	6.84	6.86	99.71
Met	1.90	4.50	4.40	102.27
Leu	6.88	9.06	9.38	96.59
Lys	9.24	10.79	11.74	91.91
His	5.44	7.90	7.94	99.50

at least; this is confirmed by the reproducibility of the peak areas when the samples were protected from light and kept at 4°C. De Jong et al. [13] have observed an instability of the di-Dns-Tyr, probably due to oxidation. Under our experimental conditions, the di-Dns-Tyr remained unaltered. The average detection limit obtained by this method is < 25 pmol.

Under the conditions described, column separation efficiency deteriorated after approximately 400 chromatographic analyses: consequently, the disposable guard-column cartridge was changed every 100 analyses.

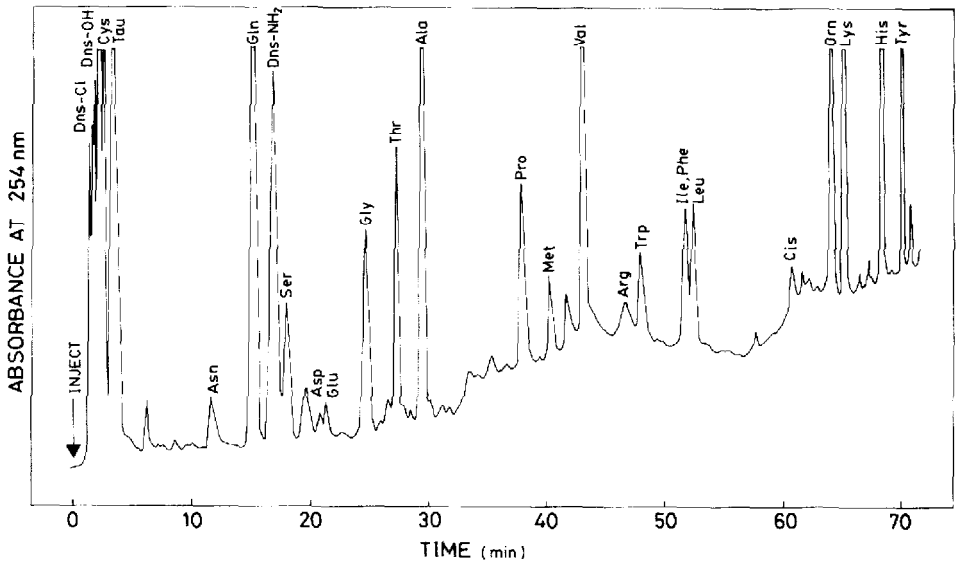

Fig. 2. Chromatogram of free Dns-amino acid derivatives in plasma of non-inoculated mouse. Conditions as in Fig. 1. Unmarked peaks were not identified.

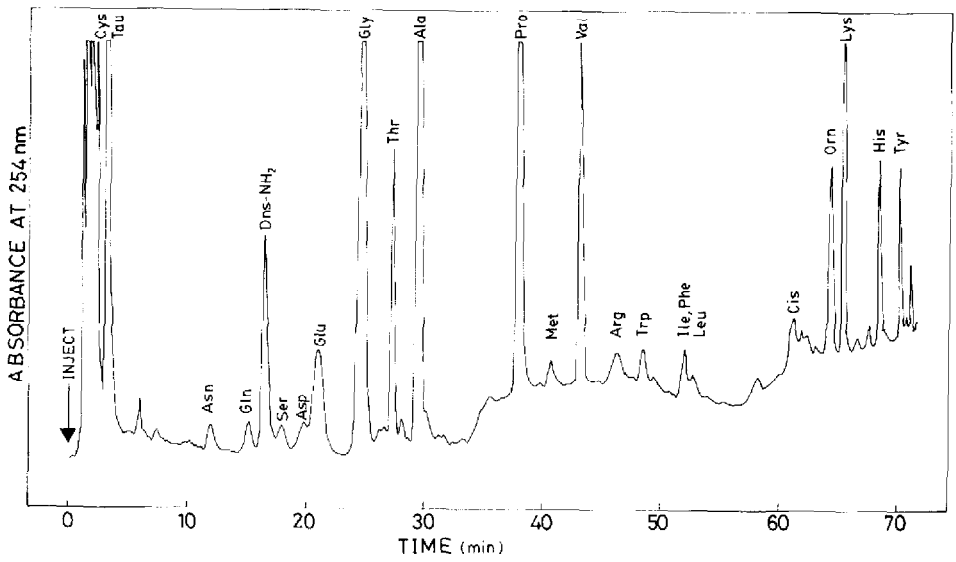

Fig. 3. Chromatogram of free Dns-amino acid derivatives in tumour ascitic liquid seven days after inoculation. Conditions as in Fig. 1.

Free amino acids in biological fluids

The chromatograms of Dns derivatives of free amino acids, in both plasma and ascitic liquid (Figs. 2 and 3), closely matched the profiles of the standard amino acid mixture (Fig. 1). Some still unidentified minor peaks appear in the biological fluid chromatograms. Albino Swiss mice are frequently used in experimental oncology, but there is very little information about the normal levels of free amino acids in the plasma of these animals [22]. Table III shows the free amino acid concentrations of the plasma of normal non-inoculated, freely feeding, mice and of the ascitic liquid of a tumour-bearing mouse at the 7th day after inoculation (the middle of the exponential phase of growth). A comparison of amino acid levels in mouse plasma reported in this study with the data recently reported for rat plasma [23, 24] showed good agreement in the relative distribution pattern of all amino acid concentrations. However, the levels of almost all amino acids in mouse plasma were considerably lower than those of rats (ca. 50% or less). Similar results were reported for free essential amino acids in human plasma [25]. The levels of these

TABLE III

AMINO ACID CONCENTRATIONS IN PLASMA OF NON-INFESTED MICE AND IN ASCITIC FLUID OF MICE INOCULATED WITH EHRLICH ASCITIC TUMOUR CELLS

The values represent the mean ± S.E.M. of six animals. Each analysis was performed in duplicate.

Amino acid	Plasma (μmol/l)	Ascitic fluid (μmol/l)
Cys	114.2 ± 6.9	130.4 ± 4.0
Tau	215.4 ± 9.5	136.4 ± 4.2
Asn	35.5 ± 1.0	8.5 ± 0.7
Gln	220.5 ± 8.1	19.6 ± 2.0
Ser	101.2 ± 3.1	25.4 ± 1.2
Asp	18.2 ± 1.0	71.7 ± 1.8*
Glu	21.0 ± 1.3	41.7 ± 3.9
Gly	196.9 ± 10.5	385.3 ± 19.8
Thr	154.3 ± 8.8	108.7 ± 3.9
Ala	289.1 ± 5.0	313.4 ± 4.3
Pro	109.2 ± 2.8	235.9 ± 4.2
Met	35.8 ± 1.3	18.0 ± 0.7
Val	155.5 ± 5.1**	30.8 ± 3.8
Arg	63.8 ± 1.3	58.4 ± 2.6
Trp	43.3 ± 2.7	17.7 ± 0.5
Ile+Phe	199.6 ± 6.6	25.4 ± 2.3
Leu	152.8 ± 1.5	19.8 ± 1.7
Cis	21.5 ± 0.8	9.6 ± 0.8
Orn	27.9 ± 1.3	22.3 ± 2.2
Lys	157.0 ± 9.9	102.7 ± 2.5
His	68.1 ± 4.1	35.9 ± 1.2
Tyr	55.5 ± 1.2	27.7 ± 0.9

*This concentration of aspartate in ascitic liquid disagrees with our previous value for aspartate determined by enzymatic analysis [20], probably due to the coelution with another not yet identified compound synthetized by the tumour.

**Derived from three determinations only.

amino acids in the rat are three times higher than those in man. The concentrations of the non-essential amino acids alanine, glutamine and glycine were highest in mouse plasma.

The results of the HPLC determinations of glutamine, glutamate, asparagine and aspartate agreed with our previous results for these amino acids determined by enzymatic analysis [20].

The literature examined contained only a few data for proline and cysteine, since these amino acids are undetected by OPA-derivatized HPLC. The secondary amines do not react with OPA; cysteine reacts with OPA only in the presence of iodoacetic acid [8]. In mouse plasma, the free amino acid concentrations ranged from 18 nmol/ml for aspartate to 289 nmol/ml for alanine. Cysteine and proline had intermediate concentrations. The ascitic liquid in the peritoneal cavity is formed by extravasation of plasma, but its amino acid pattern is very different from that of plasma. It is remarkable that glutamine was almost completely consumed, which reflects the avidity of tumour cells for this amino acid, their main source of energy. The levels of free essential amino acids: methionine, isoleucine, phenylalanine, leucine, histidine and tyrosine were lower in ascitic liquid than in plasma. Conversely, aspartate, glutamate, glycine and proline concentrations were significantly increased. These results suggest that the tumour acts as a sink for the essential amino acids required for cell growth, and that some non-essential amino acids are produced by the tumour as end-products of nitrogen metabolism. Overall amino acid movements between host and tumour are still under study.

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