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Note

Quantitation of Dns-amino acids from body tissues and fluids using high-performance liquid chromatography

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The development and refinement of high-performance liquid chromatography (HPLC) in recent years has led to considerable interest in this technique as a means for accomplishing complex separations of biologically important compounds. Standard techniques for the separation of amino acids are complex [1], costly and by comparison with HPLC, time consuming.

(5-Dimethylaminonaphthalene-1-sulfonyl chloride (Dns-Cl) reacts with both primary and secondary amino acids and the highly fluorescent derivatives have been separated using thin-layer [2] and column [3] chromatography. These derivatives provide high sensitivity and are stable over relatively long periods.

The formation of Dns-amino acids involves a series of reactions and the product yield appears to be dependent on the relative amounts of Dns-Cl and amino acid and on conditions using during incubation [4]. Careful control of reaction conditions which favor formation and inhibit decomposition of Dns-amino acids revealed the product yield to be independent of the Dns-Cl:amino acid ratio over a 1000-fold range [5]. Previous studies also emphasized the importance of carefully controlling reaction pH and temperature to optimize product yield [6]. An objection to the use of Dns-Cl for amino acid derivatization is that multiple derivatives of several amino acids may form. We have selected conditions such that formation of multiple derivatives is minimal and does not pose a problem.

Finally, pre-column derivatization requires no special equipment, can be carried out at room temperature and the resulting derivatives are stable over days permitting the use of automatic sampling devices. Considerable work has been published related to the chemistry of the reaction between Dns-Cl and amino acids [4—6]. Less information is available relating the application of the method to separation and quantitation of amino acids in biological samples.

In this paper we present a method for sample preparation, derivatization

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with Dns-Cl and quantitation of amino acids in biological tissues and fluid using HPLC.

EXPERIMENTAL

Apparatus

A high-performance liquid chromatograph (Beckman Instruments) consisting of a Model 421 microprocessor, two Model 110A pumps, a Model 210 sample injector with a 50-μl sample loop and a C-R1A recorder-integrator was used. Fluorescence was detected using a fluorometer (Gilson Spectra/Glo) with a standard flow-cell, 7-51X excitation filter and 3-72M emission filter. Prepacked reversed-phase columns (Ultrasphere-ODS, 5 μm, 25 cm × 4.6 mm) were used for separation. Reagents (water, acetonitrile and tetrahydrofuran) were HPLC grade (Baker). Dns-Cl dissolved in acetone (Pierce) was diluted to a final concentration of 6 mg/ml with acetone.

Standards

Standards were prepared by dissolving the appropriate amount of various amino acids (Sigma) in 0.5 *M* sodium bicarbonate (pH 8.5) to yield a 10 m*M* concentration. Twenty-one amino acids were prepared in this way and included taurine, asparagine, glutamine, serine, aspartic acid, hydroxyproline, glutamic acid, threonine, glycine, alanine, arginine, methionine, proline, valine, phenylalanine, tryptophan, leucine, isoleucine, lysine, tyrosine and histidine. Volumes of 20-μl of each were then mixed together in small tubes and 80 μl of sodium bicarbonate added for a total volume of 0.5 ml (0.4 nmoles of each amino acid per μl).

Extraction procedures

Although much smaller amounts of tissue can be utilized, we generally extracted the amino acids from either 1.0 g of brain or liver or 1.0 ml of serum. All extraction procedures were conducted in an ice bath and a refrigerated centrifuge. The brain and liver were homogenized in 2.0 ml of ice-cold 80% ethanol and the serum was added to this volume of 80% ethanol. The tissues were then centrifuged at 1500 *g* for 15 min. This extraction was repeated twice more and the supernatants pooled and evaporated to dryness in a vacuum oven. The residue was dissolved in distilled water (2.0 ml/g of tissue or ml of serum) and frozen until analyzed for amino acid content. Known quantities of ^{14}C-labeled amino acids were added to serum and tissue samples and put through these extraction procedures. Our recovery rates ranged from 87—93%. These are the methods of Dickerson and Pao with some modification [7].

Preparation of Dns derivatives

Aliquots of brain and plasma extract were added to equal volumes of sodium bicarbonate (0.5 *M*, pH 8.5) and mixed. Standard and samples were then derivatized together. The standard was comprised of 0.1 ml sodium bicarbonate (pH 8.5) 25 μl of the standard mixture (10 nmoles of each amino acid) and 0.1 ml Dns-Cl. Samples mixed at the same time consisted of 0.1 ml of plasma

or tissue extract in sodium bicarbonate, 25 μl of the standard mixture and 0.1 ml Dns-Cl. The pH of the standard and samples after adding Dns-Cl is 9.2—9.5. Twenty samples and 4—5 standards were derivatized for assay the following day, covered tightly and placed in the dark at room temperature overnight. Dns derivatization is complete in 3—4 h and if left overnight we have found no decrease in fluorescence. Water (0.8 ml) adjusted to a pH of 8.5 with sodium hydroxide, was then added, the samples and standard centrifuged to remove any precipitate and 50 μl injected onto the column. This volume represents 0.5 nmoles of each amino acid in both standard and sample. The presence of the internal standard prevents confusion when quantitating peaks which are not completely resolved and is especially useful when one of these peaks is absent. The 50-μl volume placed on the column contains the amino acids from 1.25 mg of tissue or 2.5 μl of plasma.

Chromatography

Resolution of the peaks was accomplished using gradient elution with the mobile phase in pump A consisting of 10 m*M* sodium acetate buffer, pH 4.18— tetrahydrofuran, (95:5) and that in pump B acetonitrile—tetrahydrofuran (90:10). The mobile phase was begun at a flow-rate of 1.0 ml/min at 10%B and increased to 40%B over 30 min at which time an isocratic hold lasting 15 min was instituted. At the end of the isocratic period B was increased to 100% over 3 min and maintained at 100% for an additional 12 min. The column was then re-equilibrated with A—B (90:10) for 8 min.

RESULTS

Separation of a standard mixture of amino acids is shown in Fig. 1. Most of the products of the Dns derivatization procedure including dansic acid and unhydrolyzed Dns-Cl elute with or very close to the solvent front. The other major by-product, Dns-amide (DAM), elutes from the column at 31 min and serves as a convenient midrun marker. Each peak in Fig. 1 represents 0.5 nmoles of the amino acid at 1/5 the maximal sensitivity of the fluorometer. Duplicate analysis of samples derivatized as described yields excellent reproducibility with variations of 0—4%. An advantage of this method is that the secondary amino acids hydroxyproline (HPR) and proline (PRO) are also derivatized and easily identified (Fig. 1).

The formation of multiple derivatives of the amino acids lysine (LYS) and tyrosine (TYR) did not occur when the pH was carefully controlled during derivatization and only the di-Dns derivatives were formed. Dns derivatization at a lower pH (8.5) results in the formation of multiple derivatives of tyrosine with a second peak eluting at 28 min (Fig. 2). Dns derivatization of the epsilon amino group of lysine may also occur if the pH is too low resulting in multiple derivatives of this amino acid. This poses no problem since ε-Dns-LYS elutes as a well defined peak after, and separated from, glycine (GLY).

A chromatogram from a liver sample showing the levels of various amino acids is shown in Fig. 3. Although some unidentified peaks are apparent they are minor and elute at times different from those of the standard. It is

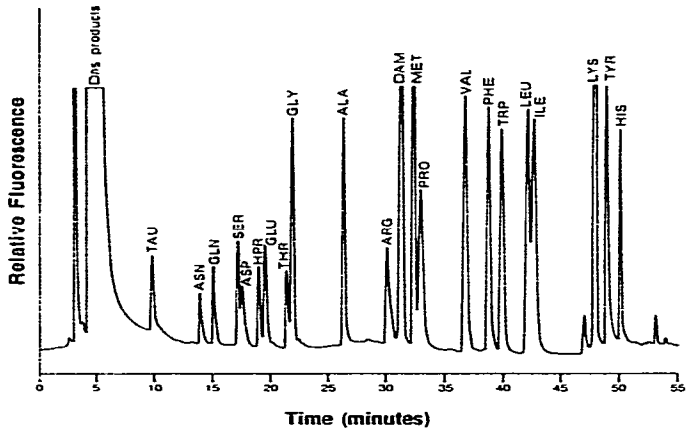

Fig. 1. A chromatogram showing the relative fluorescence and retention times of a mixture od 21 Dns-amino acids. Each amino acid peak represents 0.5 nmoles. Gradient elution, mobile phase: A = 10 m*M* sodium acetate buffer, pH 4.18—tetrahydrofuran (95:5); B = acetonitrile—tetrahydrofuran (90:10), flow-rate 1.0 ml/min. Peaks: TAU = taurine; ASN = asparagine; GLN = glutamine; SER = serine; ASP = aspartate; HPR = hydroxyproline; GLU = glutamate; THR = threonine; GLY = glycine; ALA = alanine; ARG = arginine; DAM = Dns-amide; MET = methionine; PRO = proline; VAL = valine; PHE = phenylalanine; TRP = tryptophan; LEU = leucine; ILE = isoleucine; LYS = lysine; TYR = tyrosine; HIS = histidine.

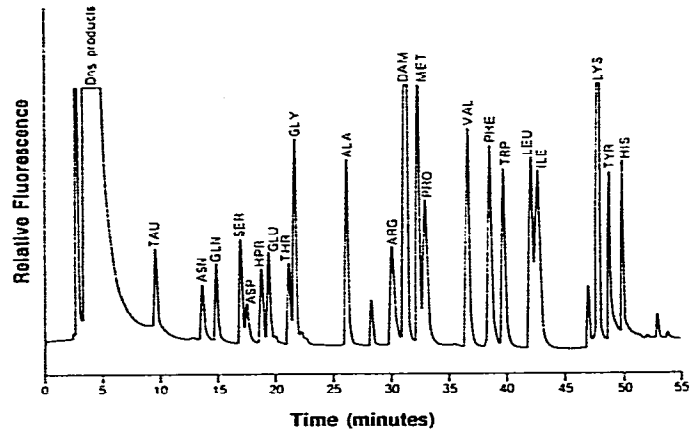

Fig. 2. A chromatogram of a standard mixture of Dns-amino acids, derivatized at pH 8.5. A second derivative of tyrosine (mono-Dns-tyrosine) elutes at 28 min. Separation procedure and abbreviations as in Fig. 1.

apparent that certain amino acids (ASN, HPR, ARG, MET, TRP and HIS) are present in small amounts or absent from this sample.

The ability to reproduce retention times is essential when accomplishing a complex separation such as a mixture of amino acids. Using the system described we have found the retention times to be very consistent from one chromatogram to the next. The retention times and variability of early, middle and late eluting peaks were determined. Taurine (TAU), valine (VAL) and lysine (LYS) were selected and the means ± S.E. for ten determinations were:

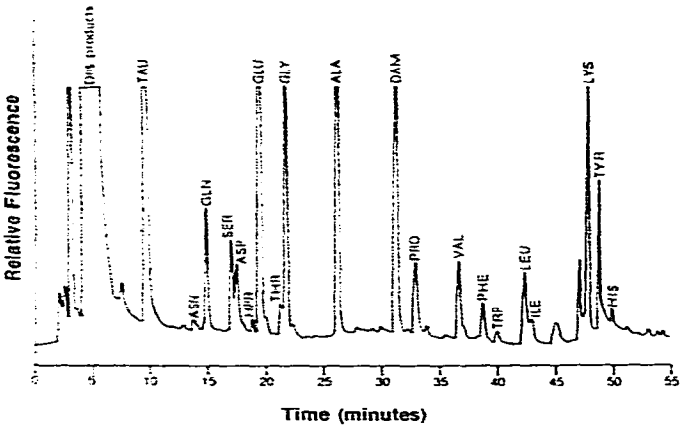

Fig. 3. A sample of rat liver chromatographed after Dns derivatization without internal standard. Separation procedure and abbreviations as in Fig. 1.

TABLE I

PERFORMANCE DATA INDICATING THE WITHIN-RUN AND RUN-TO-RUN PRECISION AS INDICATED BY COEFFICIENTS OF VARIATION (C.V.)

Concentration of each amino acid, 0.5 nmoles. In all cases $n = 6$.

Amino acid	Mean*	C.V. (%)
		Precision within-run
Glutamine	38	1.4
	42	3.9
	41	3.9
	39	1.9
	40	1.6
	40	2.2
Alanine	154	0.9
	155	0.9
	156	1.3
	155	1.2
	154	0.7
	155	1.6
Lysine	516	0.5
	506	2.8
	493	1.7
	500	1.6
	508	2.6
	514	2.1
		Precision run-to-run
Glutamine	40	3.6
Alanine	155	0.5
Lysine	506	1.8

*Mean = relative area ($\times 10^3$) computed by the integrating recorder under conditions described in the Experimental section.

TAU 9.2 ± 0.09 min, VAL 38.64 ± 0.06 min and LYS 47.5 ± 0.02 min.

Data in Table I demonstrate the precision achieved for three amino acids from the beginning, middle and end of the chromatograms. The run-to-run data were obtained over a 3—4 month period.

Even though retention times are reproducible we chose to utilize an internal standard for each peak to insure that (a) if variations in retention time occurred we could still identify the peaks and (b) if variations in the derivatization reaction occurred it would affect standard and sample to the same degree. Fig. 4 is a chromatogram of sample and standard (0.5 nmoles of each amino acid) derivatized together. Knowing the area of the standard alone (integration of peaks as in Fig. 1) and of the area of the sample + standards (as in Fig. 4) it is simple to quantitate the amount of amino acid in each peak.

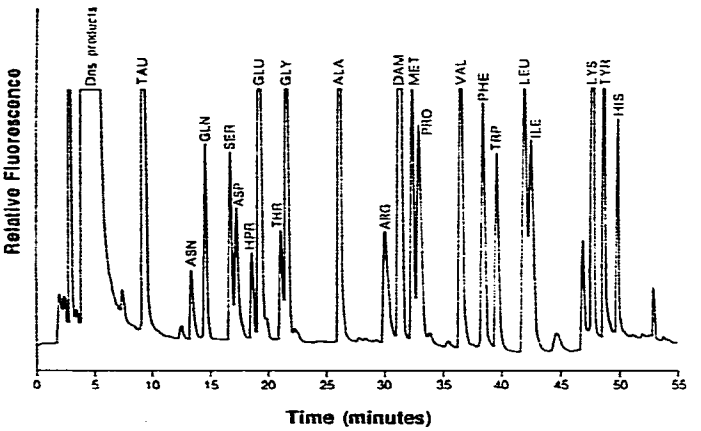

Fig. 4. Chromatogram of a sample of rat liver Dns derivatized with internal standards as in Fig. 1. The same separation procedure and abbreviations as in Fig. 1.

The figures presented here are from chromatograms obtained from liver and standards. We have used the same technique to analyze samples of brain and plasma, varying only the amount of sample derivatized or the amount injected onto the column (to prevent overloading). We observe only minor differences and find that the technique works equally well for all tissues tested thus far.

DISCUSSION

The technique for separation and quantitation of derivatized amino acids described herein provides several advantages over other methods. Pre-column derivatization with Dns-Cl is rapid and economical and in contrast to pre-column derivatization with *o*-phthalaldehyde (OPA) the product formed is very stable. We have run repeat chromatograms on derivatized samples over a 96-h period and find no detectable decrease in fluorescence. This observation is of critical importance when the use of autosamplers and large numbers of samples are involved. Pre-column derivatization with OPA precludes use of an automatic sampler since the OPA-amino acid adducts formed degrade at different rates and thus result in peak height changes with time [8]. A further

disadvantage of OPA is that it does not react with secondary amino acids such as proline and hydroxyproline [9]. More recently a system was presented which permits simultaneous detection of OPA derivatized primary and secondary amino acids [10].

The short analysis time required (55 min) is yet another advantage. Sample preparation is fast and simple which permits a technician to learn the technique and do the complete analysis at a fraction of the time and cost required by an amino acid analyzer. The ability to quantitate primary and secondary amino acids during a single run is yet another advantage of this method since the reaction between Dns-Cl and amino acids is linear and quantitative over a wide range of concentrations (up to 1000 times excess Dns-Cl) [5].

In contrast to the findings of Hill et al. [11] we encountered no problem resolving threonine and glycine in either tissue samples or plasma. In agreement with their findings we noted several peaks which were especially susceptable to changes in the concentration of buffer. A decrease in buffer concentration to 5 m*M* causes methionine and proline to co-elute and arginine to be retained longer, appearing after the Dns-amide peak. Retention of arginine is also especially sensitive to changes in pH; a decrease causes an increase in retention time while an increase moves arginine closer to alanine. The susceptability of arginine and Dns-amide peaks to changes in mobile phase composition have also been observed by others [3,12]. Serine and aspartate may also co-elute if the pH is decreased.

We have found pre-column derivatization with Dns-Cl to be highly reliable and reproducible. Repeat analyses of the same samples derivatized at various times have produced variations of less than 5%, an error which can be attributed to dilution etc. during sample preparation (Table I) and is in the range of variation observed in the separation of OPA-amino acid adducts [13].

The use of HPLC and pre-column derivatization to form fluorescent adducts can provide a useful tool for the rapid, economical separation and quantitation of amino acids in biological fluids and tissues. Extension of this technique may prove useful in peptide analysis and separation and quantitation of other biogenic amines.

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REFERENCES

1 D.H. Spackman, W.H. Stein and S. Moore, Anal. Chem., 30 (1958) 1190.
2 W.R. Gray, Methods Enzymol., 25 (1972) 121.
3 Y. Tapuhi, N. Miller and B.L. Karger, J. Chromatogr., 205 (1981) 325.
4 D.J. Neadle and R.J. Pollitt, Biochem. J., 97 (1965) 607.
5 Y. Tapuhi, D.E. Schmidt, W. Lindner and B.L. Karger, Anal. Biochem., 115 (1981) 123.
6 C. Gros and B. Labouesse, Eur. J. Biochem., 7 (1969) 463.
7 J.W.T. Dickerson and S.K. Pao, Biol. Neonate, 25 (1975) 114.
8 B.R. Larsen and F.G. West, J. Chromatogr. Sci., 19 (1981) 259.
9 M. Roth, Anal. Chem., 43 (1971) 880.

10 Y. Ishida, T. Fujita and K. Asai, J. Chromatogr., 204 (1981) 143.
11 D.W. Hill, F.H. Walters, T.D. Wilson and J.D. Stuart, Anal. Chem., 51 (1979) 1338.
12 J.M. Wilkinson, J. Chromatogr. Sci., 16 (1978) 547.
13 K. Lenda and G. Svenneby, J. Chromatogr., 198 (1980) 516.