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Short Communication

Analysis of physiological amino acids using dabsyl derivatization and reversed-phase liquid chromatography

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ABSTRACT

A method is described for the measurement of the specific radioactivity of primary amino acids in physiological samples. The amino acids were dabsylated followed by separation using high-performance liquid chromatography. We measured the concentration of amino acids from rat plasma or liver samples. Chromatographic analyses resolved phenylalanine from a mixture of amino acids in plasma within 30 min. An extended chromatographic gradient program completely separated all physiological amino acids within 75 min. This method is as sensitive as any current method of amino acid analysis and offers several advantages including (1) simple pre-column derivatization and (2) stability of derivatized samples.

INTRODUCTION

The maintenance of protein metabolism is required for cell viability and growth. Protein metabolism consists of the reactions involved in protein synthesis and degradation. Current methodologies to measure rates of protein synthesis or degradation use either stable or radioactive isotopes of amino acids. For analysis of protein synthetic rates, the incorporation of a specifically labeled amino acid into protein is measured (for review see ref. 1). The measurement of protein synthesis *in vivo* or *in vitro* depends upon the amount of labeled amino acid in the protein and the specific activity of the amino acid precursor. The major source of error is determining the specific activity of the labeled amino acid in the precursor. The direct precursor for protein synthesis is the amino acyl-tRNA. However, measurement of the specific radioactivity of the amino acyl-tRNA is difficult and time-consuming.

The specific activity of the amino acid bound to the tRNA is in equilibrium with that in the extracellular or intracellular space. Therefore, the specific activity the radiolabeled amino acid in the extracellular space is used to estimate of the precursor amino acyl-tRNA (for review see refs. 1–3). The specific radioactivity of the amino acid can be measured by a number of techniques. The specific radioactivity can be measured with an amino acid analyzer fitted with a split-stream attachment for determining the radioactivity [2]. This technique is slow and expensive. Alternatively, phenylalanine can be converted enzymat-

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ically to β-phenethylamine [3]. However, this method is somewhat difficult in that the entire assay needs to be performed in the dark and requires precise timing. Otherwise, marked variation in the measurements will occur (T. C. Vary, unpublished results). Furthermore, not all amino acids form derivatives that are easily isolated from other amino acids by phase separation techniques. To circumvent these problems, amino acids may be derivatized and separated using HPLC [4]. HPLC separation offers several advantages over other techniques in that it is relatively quick, reproducible and inexpensive.

The present study describes the use of dabsylation prior to separation of amino acids via HPLC for measurement of the specific radioactivity of amino acids from physiologic samples. Pre-column dabsylation has been used previously to examine the concentration of amino acids in urine [5]. However, in the studies by Lin and Wang [5] ammonia was found to interfere with the determination of methionine and valine. As valine is used to measure rates of protein synthesis in liver [6], the response generated by the ammonia peak would interfere with quantification of valine. Furthermore, the procedures for dabsylation of amino acids has not been utilized in samples containing a high concentration of proteins, such as plasma. These problems have been overcome in the present study, allowing for the measurement of amino acids from physiological samples by dabsylation. The procedure was used to analyze the specific radioactivity of phenylalanine in plasma and methionine in liver samples from rats.

EXPERIMENTAL

Materials

Dabsyl chloride (DABS-Cl) was purchased from Pierce (Rockford, IL, USA) and was used without prior recrystallization [7]. Acetonitrile (OmniSolve) was obtained from EM Science (Gibbstown, NJ, USA). Dimethylformamide (Certified ACS) and citric acid (anhydrous) (Certified ACS) were purchased from Fisher Scientific (Swedesboro, NJ, USA). All aqueous solutions were made up using highly purified water purchased from Fisher Scientific. DABS-amino acid kit was purchased from Beckman Instruments (Altex Division, Berkeley, CA, USA). L-[^{3}H]Phenylalanine and L-[^{35}S]methionine were purchased from Amersham (Arlington Heights, IL, USA) and ICN Radiochemicals Division (Irvine, CA, USA). L-Norleucine and an amino acid standard solution containing seventeen amino acids were obtained from Sigma (St. Louis, MO, USA). The amino acid standard was supplied at a concentration of 2.5 nmol/ml, except for cystine which was 1.25 nmol/ml, in 0.1 *M* HCl. All other reagents were of analytical grade.

High-performance liquid chromatography

The HPLC system (Beckman Instruments) consisted of two Model 110A pumps, a Model 450 system controller with integrator and a Model 150 absorbance detector (fixed wavelength). Dabsylated amino acid derivatives were separated on a 5-μm Econosphere (250 mm × 4.6 mm I.D.) HPLC column (Alltech Assoc., Deerfield, IL, USA). A 5-μm Adsorbosphere C_{18} guard column (Alltech Assoc.) was also used. Both the guard column and the analytical column were presaturated with citric acid and dimethylformamide. To accomplish this, the columns were washed with a buffer containing 100 m*M* sodium citrate (pH 3.5) containing 20% (v/v) dimethylformamide at a flow-rate of 1.0 ml/min for 1 h.

The dabsylated amino acid derivatives were separated with a flow-rate of 1.4 ml/min at room temperature. Details of the gradients used to produce the mobile phases are given in the Results section. The gradients are modifications of the ones described by Vendell and Aviles [8]. All changes in the gradient occurred in linear steps. Buffer A consisted of 10 m*M* sodium citrate (pH 6.5) containing 4% (v/v) dimethylformamide. Buffer B consisted of 30% (v/v) buffer A and 70% (v/v) acetonitrile containing 4% (v/v) dimethylformamide. Both buffers were filtered and degassed prior to use.

Preparation of dabsylated amino acids

A 500-μl standard amino acid solution (Sigma) or 250 μl of a physiologic sample (plasma) was

diluted with 2 ml of 10% (w/v) trichloroacetic acid (TCA). Liver samples (0.2 g) were homogenized (Polytron PT10, Brinkman Instruments, Westbury, NY, USA) in five volumes of 10% TCA. The sample was centrifuged at 10 000 *g* for 10 min. The supernatant was decanted and the volume measured. The pH of the supernatant was raised to 9.0 with potassium hydroxide [9,10] and the volume measured. A 40-μl sample of the neutralized extract was pipetted into a 50 mm × 6 mm glass vial containing 40 μl of 0.1 *M* sodium bicarbonate (pH 8.3). Next, 80 μl of a 4 m*M* solution of dabsyl chloride in acetonitrile was pipetted into the reaction vial. The reaction vial was sealed with a silicon stopper and heated at 70°C. The vial was mixed after 1 and 4 min. After 12 min, the reaction vial was removed from the heating block and allowed to cool at room temperature for 5 min. The samples were then diluted with 440 μl of a solution composed of 50 m*M* Na_2PO_4 (pH 7.0) and ethyl alcohol (1:1, v/v), and 50 μl were injected. In some experiments, 5 μl of a 2 m*M* stock solution of L-norleucine was added to the sample prior to addition of the TCA as an internal standard for quantification.

The stability of the DABS-Cl solution was tested by dabsylating the same sample on successive days and comparing the resulting chromatogram to one obtained with a fresh solution of DABS-Cl. The DABS-Cl solution was stable for a period of one week (data not shown). Routinely, the DABS-Cl solution was made up fresh the day the dabsylation was performed.

Measurement of radioactivity

A 1.4-ml fraction was collected in 10-ml scintillation vials containing 1 ml of water using a Retriever II fraction collector (ISCO, Lincoln, NE, USA). The fraction collector was connected to the outflow tubing of the absorbance detector. To the sample, 10 ml of liquid scintillant were added and the samples were shaken. Radioactivity was measured by liquid scintillation spectrometry using the proper corrections for quenching.

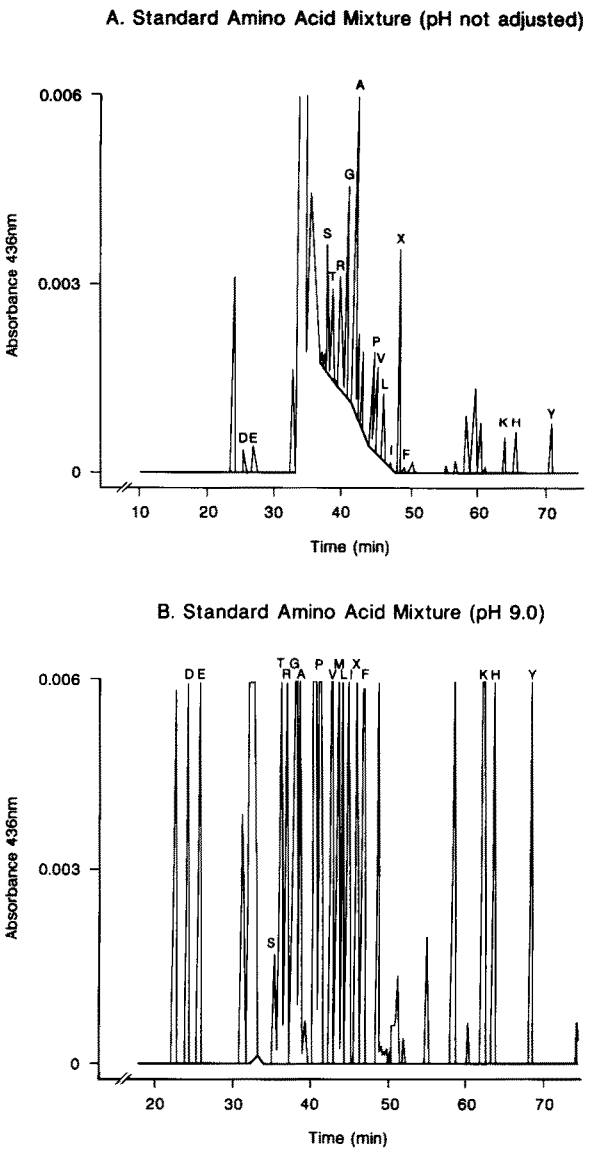

Fig. 1. HPLC separation of dabsyl derivatives from a standard mixture of amino acids: effect of sample pH. The amino acids in a standard amino mixture were either subjected to dabsylation directly (A) or the pH was increased to 9 by the addition of KOH prior to dabsylation (B). The DABS-amino acid derivatives are identified by using the one-letter abbreviation (see Table I) for the corresponding amino acid (X = norleucine). Details of the column and the gradient conditions are provided in the text. There were no peaks identified prior to approximately 20 min following injection of the dabsyl-amino acid derivatives. Routinely the integrator was turned off for the first 20 min of the chromatographic separation.

RESULTS AND DISCUSSION

Our initial goal was to examine the conditions necessary for the dabsylation of amino acids following deproteinization of proteins from plasma. To 20 μl of a mixture of seventeen amino acids of known concentrations in 0.1 *M* HCl, 20 μl of a 0.1 *M* sodium bicarbonate buffer (pH 8.3) were added. To this mixture 40 μl of the DABS-Cl solution were added. The samples were then processed as described in the Experimental section. These dabsylated amino acids were separated by HPLC using the following mobile phases. The initial mobile phase consisted of 83% buffer A and 17% buffer B. This composition was maintained for 5 min after injection of the sample. The percentage of buffer B was increased to 26% over the next 18.6 min and to 37% over the subsequent 16 min. Then, the percentage of buffer B was increased to 81% over the next 34.7 min and then to 100% over 0.3 min. After 76 min from injection of the sample, the percentage buffer B was decreased to 17%. Another sample was injected 14 min later. The results of these experiments are presented in Fig. 1A. The separation of amino acids using this protocol is similar to the results presented by Chang *et al.* [7,9,10] using a standard amino acid mixture or protein hydrolysates.

Since the dabsylation reaction is pH-dependent [8–10], with maximal derivatization occurring at pH 9.0, the pH of the standard amino acid mixture was increased to 9.0 prior to dabsylation. The results of these studies are shown in Fig. 1B. A comparison of the separation of a standard mixture of amino acids in Fig. 1A and B shows that the peak heights were enhanced in the samples where the pH was raised to 9.0 prior to dabsylation. This allowed methionine and phenylalanine to be easily quantitated.

However, 0.1 *M* HCl is insufficient to precipitate proteins in plasma. To deproteinize plasma, 250 μl of rat plasma were mixed with 2 ml of 10% TCA, and the extract was centrifuged. The pH of

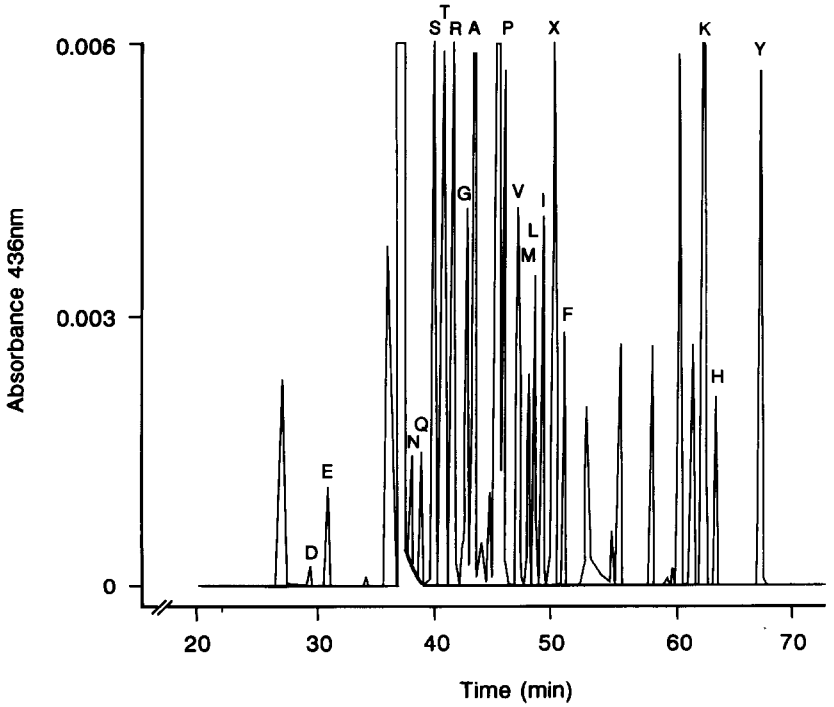

Fig. 2. HPLC separation of dabsyl derivatives from plasma amino acids. A sample of rat plasma was deproteinized with trichloroacetic acid and the pH raised to 9.0. The sample was dabsylated and chromatographed as described in Fig. 1. For peak identification see Table I.

TCA extracts was increased to pH 9.0 using potassium hydroxide. A 40-μl sample was further diluted with 40 μl of the sodium bicarbonate buffer and dabsylated. The dabsylated amino acids were chromatographed using the gradients described above. Fig. 2 shows a representative chromatogram obtained from a sample of rat plasma. As can be observed, excellent separation of seventeen amino acids present in rat plasma was obtained. This procedure gives better a resolution of amino acids in physiologic matrixes than previously reported [5]. Furthermore, there is no overlap of ammonia with any other amino acid, particularly valine and methionine.

For quantitative analysis of plasma samples, the DABS-amino acid standards were treated and dabsylated in parallel with the unknown plasma sample under identical conditions. Identification of amino acids in the standard solution or plasma sample was achieved by comparing retention times of known amounts of individual amino acids with those in the unknown samples and by coelution of amino acids added to the extracts. The retention times for the seventeen dabsylated amino acids are provided in Table I.

We quantified the amount of DABS-amino acids in our samples by two methods. When the concentration of a specific amino acid such as phenylalanine, glutamine or methionine was measured, we generated a standard curve using various amino acid concentrations. DABS-amino acids were quantitated using either peak height or peak area. The amino acid concentrations were also calculated from an internal standard (norleucine) and the known concentration of amino acids in the standard solution. The amino acid concentrations in plasma of rats measured by the DABS-Cl method and an automated

TABLE I

PLASMA AMINO ACID CONCENTRATIONS AS DETERMINED BY THE DABS-Cl HPLC METHOD AND THE AMINO ACID ANALYZER

Ion-exchange amino acid analysis of plasma samples was performed using a Beckman System 6300 amino acid analyzer as described by Vary *et al.* [14].

Amino acid	Abbreviation	Retention time (min)	Concentration (mean ± S.D.) (μmol/l)	
			DABS-Cl HPLC (n = 4)	Amino acid analyzer (n = 4)
Aspartate	D	28.7	27 ± 6	16 ± 6
Cysteine	C	30.9	N.D.	N.D.
Glutamate	E	32.7	55 ± 18	114 ± 38
Glutamine	Q	43.4	727 ± 242	563 ± 38
Serine	S	43.8	246 ± 56	233 ± 34
Threonine	T	44.6	223 ± 70	238 ± 40
Arginine	R	45.3	163 ± 24	164 ± 56
Glycine	G	46.0	210 ± 34	293 ± 32
Alanine	A	46.5	314 ± 34	526 ± 64
Proline	P	48.6	98 ± 16	214 ± 34
Valine	V	49.5	210 ± 24	177 ± 24
Methionine	M	51.0	68 ± 16	56 ± 8
Leucine	L	51.5	121 ± 18	146 ± 26
Isoleucine	I	52.2	71 ± 12	83 ± 12
Phenylalanine	F	54	47 ± 14	67 ± 14
Lysine	K	66.4	319 ± 78	390 ± 40
Histidine	H	67.6	32 ± 10	66 ± 8
Tyrosine	Y	71.5	45 ± 8	78 ± 16

ion-exchange chromatography method with ninhydrin post-column derivatization are compared in Table I.

With this basic procedure to dabsylate amino acids from physiological sources, different chromatographic programs were developed according to the speed required for analysis and nature of the amino acid analyzed. For measurement of the specific radioactivity of L-[^{3}H]phenylalanine in plasma samples, separation of the dabsylated phenylalanine was accomplished within 30 min. The chromatogram of a standard mixture of amino acids using this shorter gradient system is shown in Fig. 3A. Initially the mobile phase was 71% buffer A and 29% buffer B. The percentage of buffer B was increased over 12 min to 51%. It was further increased to 86% over the next 10 min. The percentage of buffer B was held at 86% for 3 min and then raised to 100% over 1 min. After 30 min from injection of the sample, the percentage buffer B was decreased to 29%. The HPLC system was ready for injection of another sample 10 min later. The separation of amino acids from a sample of rat plasma is shown in Fig. 3B. The plasma sample contained L-[^{3}H]phenylalanine which had been previously injected into the jugular vein of the rat [11,12]. The corresponding measurement of radioactivity in the plasma samples is shown in Fig. 3C. A single peak of radioactivity corresponding to the phenylalanine peak was observed. The specific activity is calculated from the amount of phenylalanine and the radioactivity.

Formation of the 43S pre-initiation complex in mamalian systems requires the binding of Met-tRNA^{met} to the 40S ribosome. The binding of [^{35}S]methionine to the 40S ribosome measures the rate of formation of the 43S pre-initiation complex [13]. This can be accomplished by perfusing organs with buffer containing radiolabeled methionine and measuring the incorporation of [^{35}S]methionine into the 43S pre-initiation complex [13]. The specific radioactivity of the methionine in the acid-soluble fraction of liver is required to calculate the formation of the 43S pre-initiation complex [13]. Before examining the differences in the rate of formation of the 43S pre-

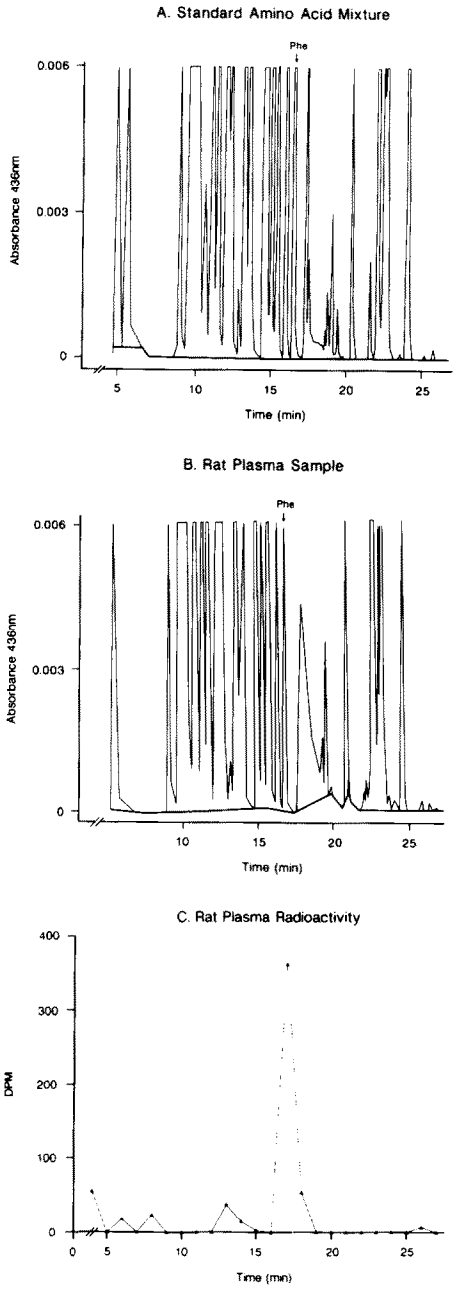

Fig. 3. HPLC separation of dabsyl derivatives from plasma amino acids: quantification of phenylalanine specific radioactivity. A sample of a standard amino mixture (A) or rat plasma (B) was mixed with 10% trichloroacetic acid, centrifuged, decanted and the pH of the supernatant raised to 9.0. The sample was dabsylated and chromatographed as described in Fig. 1. Fractions (1.4 ml) were collected in scintillation vials containing 10 ml of Liquiscint scintillation cocktail. Radioactivity in the fractions was determined by liquid scintillation spectrometry (C).

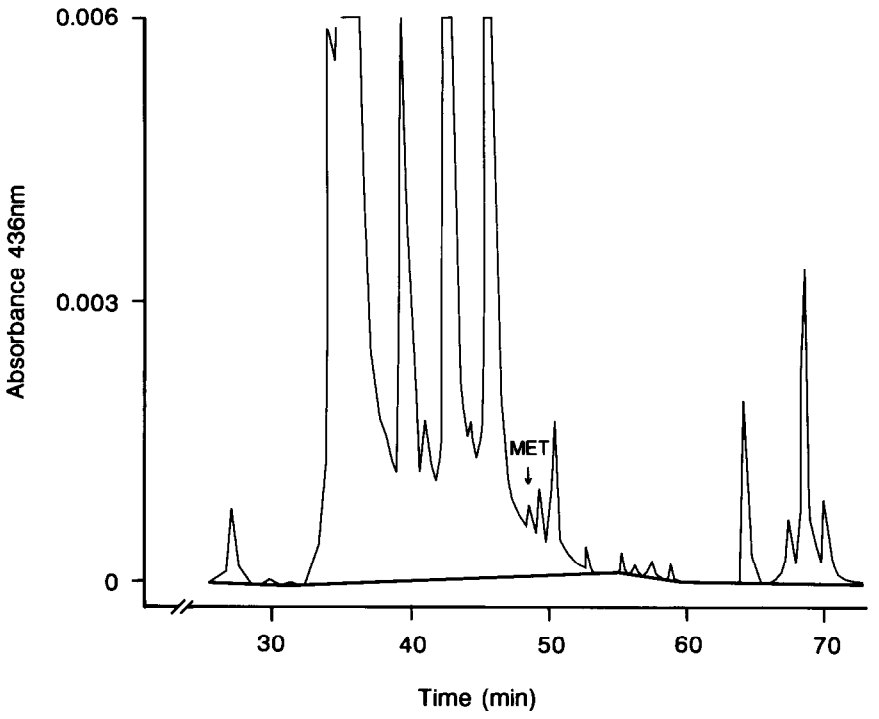

Fig. 4. HPLC separation of dabsyl-methionine in liver. Liver was frozen *in situ* with clamps precooled to the temperature of liquid nitrogen. The frozen tissue was powdered under liquid nitrogen, homogenized in four volumes of 10% trichloroacetic acid, and the pH raised to pH 9.0. The sample was dabsylated and chromatographed as described in Fig. 1. Methionine in the sample was identified by coelution with added methionine.

initiation complex in livers perfused from control and diabetic rats, we needed to develop a method to measure methionine. As can be seen from examining the chromatograms in Fig. 1B or Fig. 2, methionine was not well separated from other amino acids, making quantification difficult. To improve the separation of methionine from other amino acids, the percentage of buffer B in the initial mobile phase was reduced to 17%. This was maintained for 5 min. Over the next 18.6 min, the percentage of buffer B was increased to 26%. Next the percentage of buffer B was increased to 37% over the subsequent 15 min. Then, the percentage of buffer B was increased to 81% over the next 45 min and then to 100% over 0.3 min. After 84 min from injection of the sample, the percentage buffer B was decreased to 17%. Another sample was injected 15 min later. Livers were homogenized in four volumes of 10% TCA. A representative chromatogram from a TCA extract of rat liver is shown in Fig. 4. A single peak of radioactivity eluting at the dabsyl-methionine retention time was observed (data not shown). Measurement of the radioactivity associated with the methionine peak allowed for the calculation of the specific methionine radioactivity.

The original goal of these experiments was to develop a method to measure the specific radioactivity of amino acids from physiological samples such as plasma or tissues. This was accomplished by optimizing the sample preparation, the pre-column derivatization conditions, and the chromatographic separation. Chang *et al.* [10] have reviewed the advantages of the dabsylation procedure over other pre- and post-column derivatization procedures for measurement of amino acids in protein hydrolysates. The same advantages apply to the analysis of amino acids from physiologic samples. The biggest advantage

is that the dabsylated amino acid derivatives are stable for periods up to three months at −20°C (data not shown). The major difference between protein hydrolysates and physiologic samples is the necessity to remove proteins in the latter samples. In the present studies this was accomplished by precipitation of the plasma or tissue protein with TCA. The precipitated proteins were removed by centrifugation. It is important that the pH of the acid extract must be raised to 9.0 prior to the pre-column dabsylation procedures. Otherwise, the dabsylation reaction will not proceed.

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