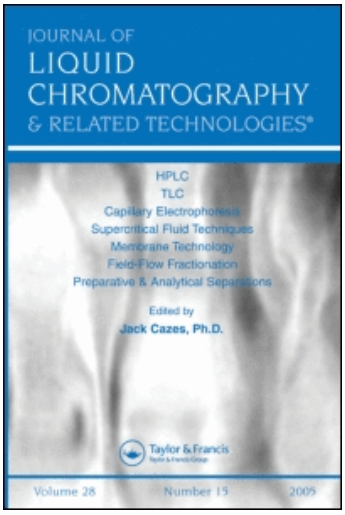

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High Performance Liquid Chromatographic Analysis for Free Valine in Plasma

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS FOR FREE VALINE IN PLASMA

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ABSTRACT

A precolumn derivatization method is presented with the use of a fluorescent derivative, 1-dimethylaminonaphhalene-5-sulfonyl-chloride, dansyl chloride, for the detection of free valine in plasma. Dansylated amino acids were determined in deproteinized samples by reverse-phase liquid chromatography. The level of detection is 100 femtomoles (10^{-15}). Sample preparation required precipitation of proteins with trichoroacetic acid and removing the excess acid with water saturated ether. The deproteinized sample was adjusted to pH 9.0 and reacted with dansyl chloride. The dansylated products were detected by ultraviolet and fluorescence spectrometry. Elution time for valine subsequent to injection is 25 minutes, while the total assay requires less than 50 minutes.

INTRODUCTION

Numerous techniques have been reported for precolumn (1-4) and postcolumn derivatization (5-7) of amino acid standards and

biological specimen. While certain advantages exist under both methodologies, precolumn derivatization is used by many investigators because postcolumn requires elaborate and most often semi-dedicated systems (7-8). Ideally precolumn derivatization reagents should have strong UV absorbance as well as fluorescence. The reaction in an aqueous media should give a single peak when chromatographed.

In this study dabsyl chloride, 4-dimethylaminoazobenezene--4 -sulfonylchloride (9): fluorescamine, 4-phenylspiro-[Furan-2-(3H). 1 -phthalan]-3,3 -dione (10) and dansyl chloride were all investigated for precolumn fluorescent derivatives. Dansyl chloride was selected as the reagent of choice.

The method described in this report employes the use of uBondapak C_{18} columns and dansyl chlorides as the detection reagent.

MATERIALS[1]

Chemicals

The following reference chemicals were purchased from Pierce Chemical Company, Box 117, Rockford, Illinois 61105. Amino Acid Standard Kit, 2.5 uM/ml in 0.1 N-HCL (Cat. No. 20088, Pierce) containing alanine, arginine, aspartic acid,

cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine and ammonia [as $(NH_4)_2$ SO_4]; Dansyl AMAC Kit, 100 mg each of the preceding amino acids (same as 20088) plus asparagine, hydroxyproline, norleucine, norvaline, and tryptophan. Dansyl chloride, 1 gram in 10 ml acetone. Cheng-Chin Polyamide Sheets, 15 x 15 cm (Cat No 20270 Pierce). Sodium Carbonate Buffer, 0.5 mole/liter, adjusted to pH 8.99 $\pm$ 0.02. Trichloroacetic acid, 5% w/v and HPLC grade Methanol were purhcased from Fisher Scientific Co. Silicone concentrate (Siliclad) from Clay Adams, Parsippany, N.J. 07054.

HPLC

A Waters Associates Model ALC 204 Liquid Chromatograph equipped with a Model 6000A Solvent Delivery System, a Model U6K Loop Injector, a Model 660 Solvent Programmer, a Model 440 Absorbance Detector, and (2) uBondapak C_{18} columns (4 mm x 30 cm) in tandem were used for the study. A Model A-4 Fluorescence Detector with a 10 ul Flow Cell (Farrand Optical Co., Valhalla, N.Y. 10951) was connected in series with the absorbance detector to the outlet of the column. The outputs of these detectors were connected to a Houston Instrument Chart Recorder. Two solvent systems were used. Pump A - 0.01 mole/liter sodium phosphate buffer pH 7.9, Pump B-HPLC grade methanol.

The flow rate was 1.2 ml/min, and all separations were carried out at 25°C. The UV detector was set at 365 nm, the fluorescence detector at 360 nm (7-37 Filter) for the primary (excitation) and 480 nm (3-72 Filter) as the secondary (emission) wavelength. A Perkin-Elmer (Hitashi 200) spectrophotometer was used for absorbance scans.

All glassware involved in the dansyl chloride reactions must be siliconized to prevent adherence of the dansyl derivatives to the glassware. All glassware was immersed into a 1% siliclad solution for several minutes, followed by a distilled water rinse and then dried at 100°C for one hour.

METHODS

Standards

A 2.5 uM/ml amino acid mixture was diluted 1:10 (0.25 uM/ml) with distilled water. A sample volume of 200 ul of the diluted standard was mixed with 300 ul sodium carbonate buffer pH 9.0 and reacted with an equal volume of dansyl chloride solution diluted to 5 mg/ml in acetone. The reaction was allowed to proceed to completion in the dark at 50° C for one hour.

Biological Samples

Biological samples were obtained from apparently healthy laboratory volunteers. Blood was collected in heparinized test tubes and chilled on ice immediately. Plasma was obtained by centrifugation at 3000 g and frozen at -40°C until analyzed.

To a series of test tubes 100 ul of plasma, blank (sodium bicarbonate buffer) and amino acid mixture was added followed by 900 ul of ice cold 5% TCA. The mixture was vortexed and centrifuged at 1800 g. The TCA was removed by five extractions with equal volumes of water-saturated ether. A 200 ul extract was added to 300 ul of sodium bicarbonate buffer, mixed, and diluted to 1000 ul with dansyl chloride. The reaction was allowed to proceed as described previously.

RESULTS

Chromatography Separations.

As shown in Figure 1, the first stage of separations of dansyl amino acids were accomplished on two uBondapak C_{18} columns in tandem. Dansyl valine was separated from the other dansylated amino acids. A twenty microliter sample (as prepared in methods "standards"), was injected on column. Flow rate was 1.2 ml/min. Operating pressures ranged between 3000-3300 PSI. A linear gradient separation was achieved with an initial solvent phase of 30% methanol - 70% of 0.01 M K_2PO_4,

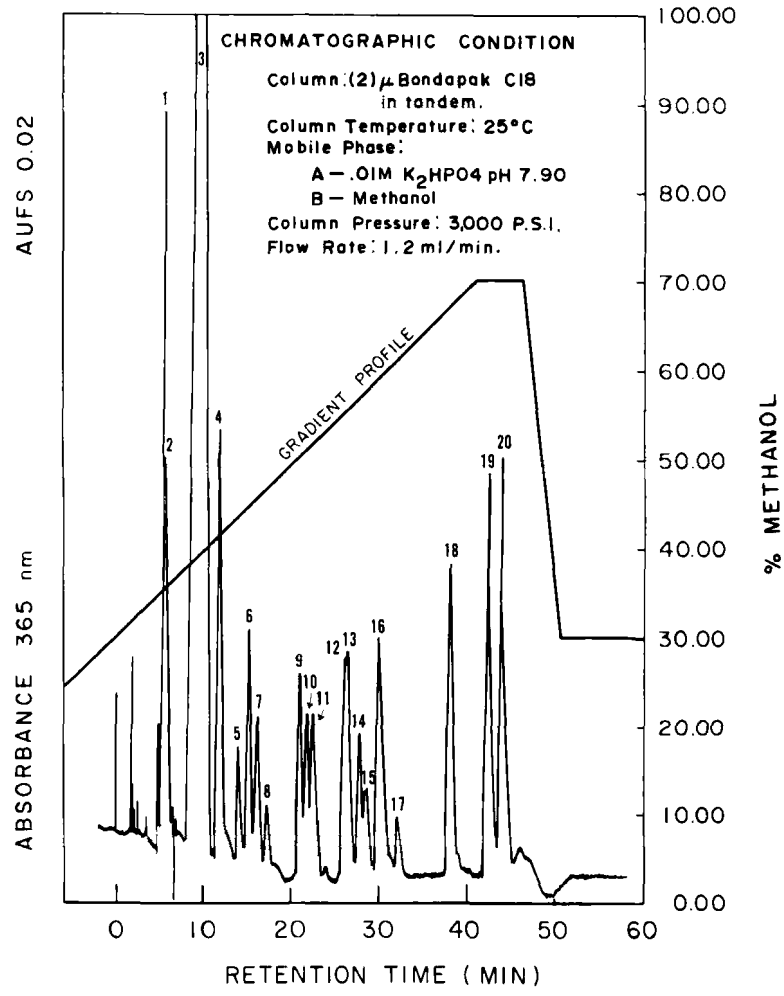

Figure 1. Gradient elution separation of dansyl amino acids. A 20 ul injection of a dansylated amino acid mixture (0.25 uM/ml). The peaks correspond to (1) asparagine, (2) glutamic acid, (3) dansyl hydroxide, (4) unknown, (5) serine, (6) threonine, (7) alanine, (8) unknown, (9) proline, (10) valine, (11) methionine, (12) cysteine, isoleucine, (13) histidine, arginine, and leucine, (14) lysine, (15) phenylalanine, (16) dansylamide, (17) unknown, (18) unknown, (19) tyrosine, (20) unknown.

pH 7.90 ± 0.05, followed by a final solvent phase of 70% methanol - 30% buffer over a 40 minute period (see Figure 1). To identify the specific retention time of each dansyl amino acid on column, two specific methods were used. In the first, each dansylated amino acid was injected on column and retention time recorded. See Table 1, for results.

In the second method the standard amino acid mixture was reacted with dansyl chloride as described in the methods. In each run, a 20 ul sample of the reacted mixture and 10 ul of one of the pure dansylated amino acids (Dansyl AMAC/Kit - each diluted in ethanol) were injected on column. Each peak was identified by the addition method and retention time. In all cases the chromatographic conditions were carefully observed (See Chromatography Separations) to insure reproducible retention times for each standard.

Thin Layer Chromatography Studies

The techniques described by Airhart (11) were applied to plasma samples to identify valine. Biological samples were

TABLE 1. Retention Times of Dansyl Amino Acids

Amino Acid	RT (Min)	Amino Acid	RT (Min)
Asparagine	5.6	Cystine	26.8
Glutamic Acid	6.0	Isoleu ine	26.8
Dansyl Hydroxide	10.0	Histidine	27.6
Serine	15.2	Arginine	27.6
Threonine	16.4	Leucine	27.6
Alanine	17.4	Lysine	28.8
Proline	22.0	Phenylalanine	28.8
Valine	22.8	Dansylamide	29.0
Methionine	23.2		

prepared as described in the methods and the dansylated amino acids chromatographed. The fraction corresponding to proline and valine, retention time 22.0 to 23.0 minutes, was collected and brought to dryness under vacuum at 50°C. The extract was reconstituted with 10 ul of ethanol and chromatographed on TLC polyamide sheets (5 x 5 cm). The two amino acids, proline and valine, were identified by direct comparison to standards.

Semiquantitative Measurements of Valine

Chromatography of valine in plasma was achieved with a gradient elution separation as shown in Figure 2, plasma valine was well separated within 25 minutes, contamination of dansyl valine from other amines was not a problem. The peak was identified by retention time, addition method and thin layer chromatography (12).

To determine the concentrations of endogenous valine in plasma the following formula was used:

$$C_x = C_s \times \frac{P_x}{P_s}$$

Where:

Cx = Concentration of valine in the plasma sample.
Cs = Concentration of valine in the standard mixture.
Px = Peak height of valine derivative from plasma.
Ps = Peak height of valine derivative obtained from the reference standard mixture (0.25 uM/ml).

Overall recoveries of aqueous standard valine concentrations were compared to spiked plasma samples (see Figure 3).

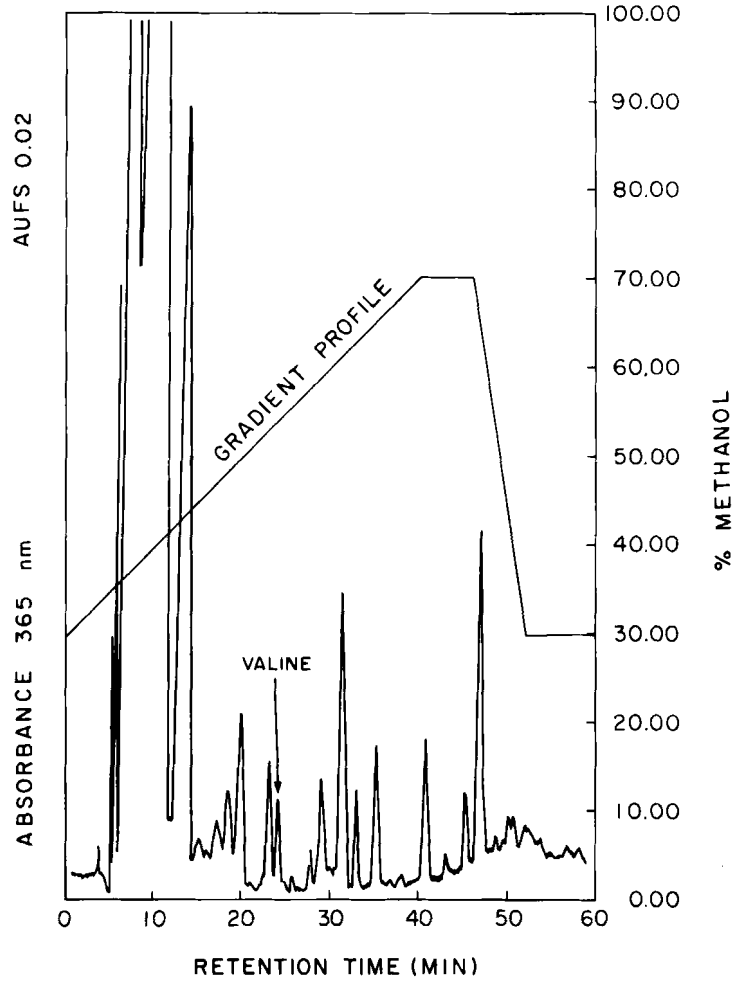

Figure 2. Gradient elution separation of plasma valine. A 100 ul injection of a dansylated plasma extract. Pooled human plasma deproteinized with trichloroacetic acid, extracted and reacted with dansyl chloride. Chromatographic conditions: see Figure 1.

As can be seen from Figure 3, better than 88% of the plasma valine was recovered when concentrations between 1.0 to 10.0 uM/ml were directly compared to aqueous standard valine

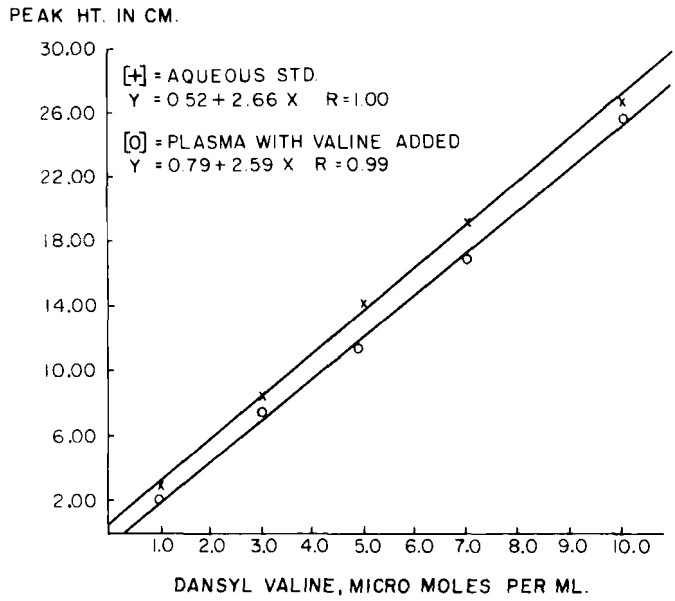

Figure 3. Aqueous standard curve containing different concentrations of valine. Samples of pooled human plasma spiked with valine. Both aqueous and plasma samples were deproteinized, extracted, reacted and chromatographed. Plasma points were corrected for endogenous valine. Points-peak height in cm, 100 ul sample injected on column, UV at 365 nm, sens, range 0.02. Chromatography conditions: see Figure 1.

concentrations. The regression equation of the aqueous standards was $Y = 0.52 + 2.66X$ with a coefficient of determination of $R^2 = 1.00$, for plasma $Y = -0.79 + 2.59X$ with a coefficient of determination of $R^2 = 0.98$ (12).

Normal Range for Endogenous Valine in Plasma

The new procedure was applied to seven fasting plasma samples. Valine concentrations determined by the HPLC procedure for a group of apparently healthy laboratory

technicians were compared to reported literature values (13) (see Table 2).

DISCUSSION

A method is described for using HPLC to determine endogenous valine in 100 ul of human plasma. It differs from other chromatography techniques in that it requires a non-dedicated HPLC system. It involves an easy sample preparation and measures valine within a reasonable amount of time (25 minutes).

A semiquantitative determination of valine in plasma is presented. It is considered as such because an external standard is used rather than an internal standard. The normal mean values calculated with this technique (250 ± 50 uM/l) were in good agreement with published normal means (252 ± 37 uM/l).

Valine was detected by absorbance and fluorescence. Absorbance is used because valine is present in plasma in large amounts. However, fluorescence can be used just as easily

TABLE 2. Comparison of Fasting Valine Levels (umole/liter) of Present Method Versus Published Levels.

Present Method		Published Levels	
N*	Mean ± SD	N	Mean ± SD
7	250 ± 50	90	252 ± 37

*Number of Measurements

under several conditions. One, smaller amounts of sample need to be injected on column (see Figure 4). Two, the dansyl derivative to be analyzed is present in smaller quantities (less than one nM/ml). Using fluorescence dansyl valine quantities were detected in the range of 100 x 10^{-15} moles.

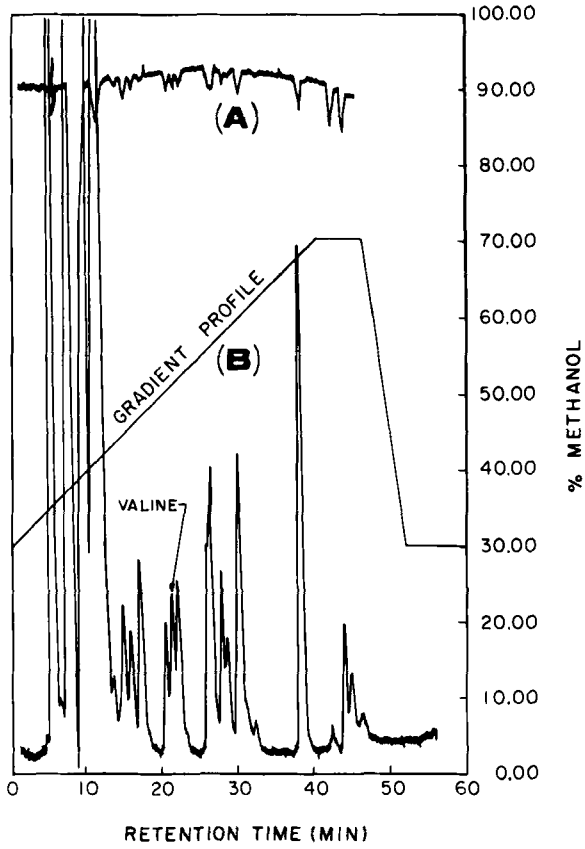

Figure 4. Comparison of absorbance versus fluorescence. A 10 ul standard mixture (0.25 uM/ml) injected on column. A - Ultraviolet absorbance chromatogram of a standard sample: abs. 365 nm, sens. 0.02. B - Fluorescence chromatogram of the same sample; exec. 360 nm, emis. 480 nm, range 0.3, aperture 5. Chromatographic conditions: see Figure 1.

The present assay lends itself ideally to the study of essential l-amino acids, either as mixtures or individually in body fluids. A practical application of the assay has been the study of induced insulin release from intravenous bolus doses of individual amino acids in subjects (13).

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FOOTNOTES

1. The manufacturer's names and products are given as scientific information only and do not constitute an endorsement by the United States Government.