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RAPID METHOD FOR DETERMINING DESMOSINE, ISODESMOSINE, 5-HYDROXYLYSINE, TRYPTOPHAN, LYSINOALANINE AND THE AMINO SUGARS IN PROTEINS AND TISSUES

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SUMMARY

A rapid and sensitive chromatographic method is described for determining desmosine, isodesmosine, 5-hydroxylysine, tryptophan, lysinoalanine, glucosamine and galactosamine at picomole levels in protein and tissue hydrolysates. This method uses either an automated amino acid analyser with a 17.5 × 0.28 cm microcolumn packed with 6.0 ± 0.5 μm spherical resin, thermostated at 52°C, one buffer system (0.21 *M* sodium citrate, pH 5.125) and 3-nitrotyrosine as the internal standard, or conventional instruments using the same system but with larger diameter columns and resins (11.0 ± 1.0 μm).

This method should be especially valuable for determining collagen and elastin in tissue hydrolysates from the amounts of 5-hydroxylysine, and desmosine or isodesmosine present, respectively, and for studying protein hydroxylation, glycosylation, cross-linking formation, and the turnover rates of collagen and elastin in normal and diseased tissues.

INTRODUCTION

Desmosine and isodesmosine, which were discovered by Partridge et al. [1] and Thomas et al. [2], are the two main lysine-derived cross-linking basic amino acid residues that stabilize mature elastin (reviewed in refs. 3 and 4). Since the levels of desmosine and isodesmosine in elastin appear to be constant among the various mammalian species studied [5], these unique cross-linking amino acids may be used to determine mature, insoluble elastin in tissues [6,7]. In addition, 5-hydroxylysine [Lys(5-OH)], which is a unique basic amino acid found in collagen and collagen-like proteins [9], has been used as a marker for determining the collagen content of various tissues [6—8, 10].

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Quantification of these unique compounds at trace levels has attracted considerable attention recently, since elastin cross-linking defects and low levels of Lys(5-OH) in collagen [11] have been implicated as part of the underlying pathology in certain heritable disorders of connective tissue such as Marfan's syndrome [11], certain types of the Ehlers-Danlos syndrome [12], and possibly in atherosclerosis [13]. Similarly, low levels of Lys(5-OH) and structural collagen defects have also been reported recently as prime factors in certain muscular dystrophies [14].

Although several methods have been described for determining these unusual amino acids in protein and tissue hydrolysates [15], and a sensitive high-performance liquid chromatographic (HPLC) procedure [16] and specific individual radioimmunoassays are available for assaying desmosine [5, 17], complete separation of all these amino acids has not been achieved.

Tryptophan is another important biological constituent of many proteins and an essential amino acid in the human diet. Free levels of tryptophan in cellular extracts and various areas of the brain are of great clinical importance since this aromatic amino acid participates in the regulation of brain serotonin metabolism. It has been implicated in various neurological and psychiatric disorders (reviewed in ref. 18). A highly sensitive HPLC method has been developed for assaying these tryptophan metabolites in physiological fluids [19]. Tryptophan, however, is readily destroyed during the acid hydrolysis (6 *M* hydrochloric acid) of proteins and tissues, and its quantitative determination has remained a difficult analytical problem. Several methods have been developed for its determination [20—23]. The most effective method to date, which is applicable to both purified proteins and tissues, is the modified alkaline hydrolysis procedure of Hugli and Moore [23] which allows quantitative recoveries (96—100%) of tryptophan in both protein and food hydrolysates, even when carbohydrates are present [24]. Other cation-exchange columns of various types, using a variety of conditions, have also been described [21, 25]. These simple chromatographic systems are not, however, without their limitations. Their resolving power and sensitivity are relatively low, and separate analyses are required for a complete determination. Extensive overlapping of some of the eluting basic amino acids occurs particularly between tryptophan and the amino sugars glucosamine (GlcN) and galactosamine (GalN), and between the diastereoisomers of 5-hydroxylysine and lysinoalanine, a very toxic compound which forms in proteins and foods by the cross-linking of lysine and dehydroalanine after alkali treatment [26].

To study further the significance of these biological processes in situ, this paper describes the development of a rapid and highly sensitive chromatographic method for the complete separation not only of the aromatic amino acids but also the complete resolution of isodesmosine and desmosine, the diastereoisomers of 5-hydroxylysine, the amino sugars GlcN and GalN, and lysinoalanine in a single chromatographic determination. The internal standard selected to test the accuracy of the chromatographic procedure is 3-nitrotyrosine [27], which elutes between phenylalanine and isodesmosine.

This rapid method is designed to be used with high sensitivity, fully automated amino acid analysers, as well as with conventional instruments, and

has been successfully applied to the determination of all of the above compounds in 1.25 h at picomole levels in a variety of tissues and proteins, including foods and feedstuffs [28—30].

EXPERIMENTAL

Materials

The four different types of cation-exchange spherical resins chosen for these studies include types DC-6A (11.0 ± 1.0 μm; lot No. 3280), DC-4A (9.0 ± 0.5 μm; lot No. 750) and DC-5A (6.0 ± 0.5 μm; lot No. 746), which were obtained from Dionex (Sunnyvale, CA, U.S.A.), and type W-3H (9.0 ± 0.5 μm), which was obtained from Beckman Instruments (Palo Alto, CA, U.S.A.). Connaught's hydrolysed potato starch, used for gel electrophoresis, was purchased from the Connaught Medical Research Labs. of the University of Toronto (Toronto, Canada). 2-Propanol was obtained from Caledon Labs. (Georgetown, Canada). Bovine *Ligamentum nuchae* elastin was purchased from Sigma (St. Louis, MO, U.S.A.). The unusual amino acid standards were obtained as follows: the diastereoisomer mixture of 5-hydroxy-DL-lysine and *allo*-5-hydroxy-DL-lysine, D-glucosamine·HCl and D-galactosamine·HCl from Calbiochem-Behring (La Jolla, CA, U.S.A.); 3-nitro-L-tyrosine from Aldrich (Milwaukee, WI, U.S.A.); lysinoalanine [N^G-(DL-2-amino-2-carboxyethyl)-L-lysine] from Miles Analytical Labs. (Elkart, IN, U.S.A.); L-tryptophan from Schwarz/Mann (Orangeburg, NY, U.S.A.); type II standard amino acid calibration mixture from Beckman Instruments. Fisher high-temperature bath oil was obtained from Allied Fisher Scientific (Fairlawn, NJ, U.S.A.). All reagents and buffers were made with high-purity laboratory water prepared by one of the procedures described by Ganzi [31] using activated carbon beds, mixed ion exchangers, glass distillation and further deionization steps. All other chemicals and reagents were of the highest purity commercially available and were used without further purification.

Methods

All of the unusual amino acid standards were supplied as chromatographically homogeneous compounds and were used without further purification. The preparation of amino acid calibration standards employed for peak identification and standardization of the instruments was carried out as previously described [33]. However, 3-nitrotyrosine, selected as the internal standard, contained considerable amounts of impurity (10%) that coeluted with histidine and ammonia. These were removed by gel chromatography on a 100 × 1.5 cm column of Sephadex G-10 equilibrated and eluted with 0.01 *M* hydrochloric acid as described by Riordan and Giese [27]. Fractions of 3.0 ml were collected at a flow-rate of 8 ml/h. The fractionation was monitored by absorbance measurements at 428 and 340 nm (A_{428}/A_{340}) and at 345 nm (ϵ = 2910). The appropriate fractions (orange—yellow) were pooled, dried in vacuo (45°C) and stored at −20°C. Desmosine and isodesmosine were isolated from bovine *Ligamentum nuchae* elastin by the preparative method described previously [7]. The epimerization of 5-hydroxylysine in 6 *M* triply glass-distilled hydrochloric acid at 110 ± 1°C for 96 h was carried out as described previously [33].

Preparation of citrate buffers

The 0.21 *M* sodium citrate buffer (20.58 g/l; J.T. Baker, Phillipsburg, NJ, U.S.A.) containing isopropanol (20 ml/l), octanoic acid (0.1 ml/l), liquefied (J.T. Baker) phenol (1.0 ml/l) and high-purity laboratory water [31] was prepared in 4-l quantities, using Pyrex solution bottles, and purified on a 45 × 0.9 cm Dionex DC-6A column (H^+ form) at 65°C (45 ml/h) as described by Hirs [34]. The purified buffer was then adjusted to either pH 5.125 or 4.775 ± 0.002 at 25°C with 10 *M* sodium hydroxide or constant-boiling triply glass-distilled hydrochloric acid (6 *M*) on a Radiometer digital pH meter (type PHM52b) equipped with a GK2302B silver chloride combination glass electrode (Radiometer A/S, Copenhagen, Denmark).

Procedures for amino acid analyses

Amino acid analyses were carried out on either a Beckman Model 120C amino acid analyser modified for accelerated chromatography to accommodate both the 0.6- and 0.9-cm diameter columns according to Spackman [35], or on a fully automated amino acid analyser (equivalent to Beckman Spinco Model 121MB) updated with 0.28-cm columns, and a linear colorimeter of 12 mm optical path-length flow cell as described by Liao et al. [36] and Hare [37]. The automated instrument was equipped with the Vista 402 automated chromatographic data reduction system (Varian, Walnut Creek, CA, U.S.A.) so that the linear signal outputs (ratios 570:690 and 440:690 nm) of the photometer could be interfaced with channel 3 (designated primary; 570 nm) and channel 4 (designated secondary; 440 nm) of the Vista 402. This has served two functions: to introduce an auxiliary signal amplifier between the linear outputs of the photometer and the Vista 402 printer—plotter, so that the sensitivity of the automated instrument increased, and to automate and accurately compute peak area and amino acid concentrations. The sensitivity of the updated instrument was also enhanced by increasing the temperature of the reaction coil bath from 100 to 129 ± 0.1°C, using mineral oil instead of water, and by shortening the ninhydrin reaction coil (0.015 cm I.D.) from 8 to 4 m. The temperature of the mineral oil bath was maintained at ±0.1°C by introducing a proportional temperature controller to the heater, to avoid baseline fluctuations during the run.

Standard preparative procedure

On a Beckman Model 120C analyser, a standard 27 × 0.9 cm column of Dionex type DC-6A resin, maintained at 47.5°C, was eluted with 0.21 *M* sodium citrate buffer (pH 5.125) at a flow-rate of 50 ml/h and a pressure of 20 bar (300 p.s.i.). These unusual amino acids have been determined from concentrated hydrolysate samples (0.2–5.0 mg) so that reasonably sized peaks for these components could be obtained. However, the regular ninhydrin reagent was pumped into the effluent stream at 25 ml/h after emergence of the neutral and acidic amino acids.

Accelerated method

The development of the 21 × 0.6 cm column of Dionex DC-6A resin was carried out with 0.21 *M* sodium citrate buffer (pH 4.775) at a flow-rate of

30 ml/h (pressure = 27 bar, 400 p.s.i.). Two temperatures were used in the analysis: equilibration at 52°C changed to 63°C at sample injection time (0 min). The calibration standards used for peak identification and standardization of the instrument were 31.5 nmol per application (250 μl). Since sample loads equivalent to 0.2—1.0 mg of protein have been applied to this column, the ninhydrin reagent was introduced into the effluent stream at 15 ml/h after elution of the acidic and neutral amino acids.

Rapid analytical procedure

On a fully automated amino acid analyser (equivalent to Beckman Model 121MP), a 17.5 × 0.28 cm microcolumn of Dionex type DC-5A resin was eluted with the same sodium citrate buffer (pH 5.125) and at the same temperature (52°C) but at a buffer flow-rate of 5.75 ml/h and a pressure of 40 bar (600 p.s.i.). For standardization of the instrument, 0.500 nmol per 100 μl calibration standard was applied when the absorbance range of the colorimeter control units was set on the 0.5 scale for both channels (570 and 440 nm). For analytical work, 100—150 μg of protein hydrolysates, which yield picomole levels of these unusual amino acids, were applied to the microcolumn. After emergence of the neutral and acidic amino acids, the ninhydrin reagent was pumped into the effluent stream at a flow-rate of 5.6 ml/h. Both the ninhydrin reagent and buffer were kept under nitrogen pressure at 2°C. The buffer line passed through a boiling water bath for degassing into a debubbler before entering the microcolumn. Regeneration of the microcolumn (4 min with 0.2 *M* sodium hydroxide containing 0.25 g/l EDTA) and equilibration (30 min with the eluting buffer) were also fully automated.

RESULTS AND DISCUSSION

The separation of isodesmosine, desmosine, the diastereoisomers of 5-hydroxylysine, the amino sugars glucosamine and galactosamine, lysinoalanine and the aromatic amino acids was carried out under a variety of chromatographic conditions in order to obtain optimal resolution in a minimum of time using instrumentation commonly available for amino acid analysis. It was found that shorter columns packed with 8% cross-linked cation-exchange spherical resins of small particle size and uniform size distribution not only permitted accelerated eluent linear flow velocities on standard amino acid columns of 0.9 cm diameter, but also exhibited high resolving power for separating all these compounds in a single chromatographic analysis. Preliminary chromatography conducted at various temperatures (30—50°C, pH (4.0—7.0) and sodium ion concentration (0.15—0.4 *M*) of the eluting citrate buffer indicated that elution of a 27 × 0.9 cm Dionex DC-6A column at 47.5°C with 0.21 *M* sodium citrate buffer, adjusted to pH 5.125, and an eluent linear flow velocity of 1.31 cm/min gave the best separation. As may be seen on the chromatogram of Fig. 1, isodesmosine is completely separated from desmosine, tryptophan is eluted between desmosine and glucosamine, and the diastereoisomers of 5-hydroxylysine and lysinoalanine emerged as discrete peaks. Excellent separation of the amino sugars was also obtained in this system. This procedure is very simple to use either as an analytical or preparative method, and a complete analysis of all these unusual basic amino acids is carried out in 2.5 h.

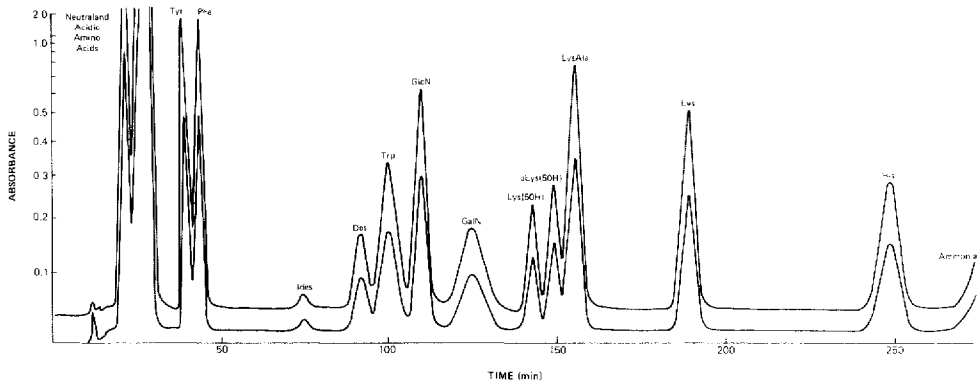

Fig. 1. Separation of a synthetic calibration mixture of eleven amino acids (250 nmol each) on a standard 27 × 0.9 cm column of Dionex type DC-6A spherical resin. The column was eluted at 47.5°C with 0.21 *M* sodium citrate buffer (pH 5.125) at a flow-rate of 50 ml/h. The upper curve shows the absorbance at 570 nm and the lower curve the absorbance at 440 nm. Peaks: Ides = isodesmosine; Des = desmosine; GlcN = glucosamine; GalN = galactosamine; Lys(5OH) = 5-hydroxylysine; aLys(5OH) = *allo*-5-hydroxylysine; LysAla = lysinoalanine.

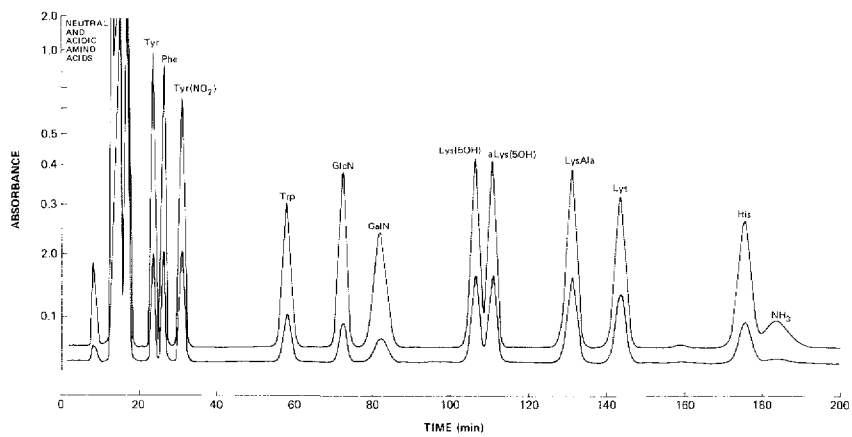

Fig. 2. Chromatographic separation of a synthetic calibration mixture of (31.5 nmol) basic amino acids on a 21 × 0.6 cm column of Dionex DC-6A resin developed with 0.21 *M* sodium citrate buffer (pH 4.775) at 30 ml/h; column equilibration at 52°C changed to 63°C at sample injection time (0 min). The upper curve shows the absorbance at 570 nm and the lower curve the absorbance at 440 nm. Peaks: Tyr(NO_2) = 3-nitrotyrosine; GlcN = glucosamine; GalN = galactosamine; Lys(5OH) = 5-hydroxylysine; aLys(5OH) = *allo*-5-hydroxylysine; LysAla = lysinoalanine.

Significant improvements in both the speed and sensitivity of this method were achieved by reducing the column diameter and volume, and by increasing the eluent linear flow velocity. The chromatographic separations shown in Fig. 2 are typical of those obtained on a 21 × 0.6 cm column of Dionex type DC-6A resin and the Beckman Model 120C amino acid analyser modified for accelerated chromatography according to Spackman [35]. The column was eluted with 0.21 *M* sodium citrate buffer (pH 4.755) at a buffer linear flow velocity of 1.77 cm/min. The initial temperature was 52°C, and increased to 65°C over 50 min. As shown in Fig. 2, each of the eleven basic components

of a synthetic calibration mixture (31.5 nmol) applied to the column emerged as a sharp, well separated peak. The major advantages of this single-column procedure over other methods are: first, the high resolving power of this system and baseline separations of the eluting amino acids and, second, that nanogram amounts of these unusual basic amino acids can be determined accurately in a single analysis in less than 2 h, using 3-nitrotyrosine as the internal standard [27]. Although 3-nitrotyrosine has not been widely employed as an internal standard, it has the added advantage that it elutes after phenylalanine and precedes all other basic amino acids. Like norleucine, 3-nitrotyrosine does not occur naturally. It is stable to acid hydrolysis for at least 96 h at 110°C, it reacts with ninhydrin, and its colour yield is 1.01 relative to 1.02 for valine [27]. This compound, however, is rather unstable to alkaline hydrolysis and should be added after the neutralization step. 5-Methyltryptophan has been used as an internal standard for tryptophan determination using alkaline hydrolysis [23], but its elution position has been reported to be sensitive to minor changes in the pH, ionic strength and temperature of the eluting buffer [25]. Another application found for this single-column system is in the determination of the hydroxyprolines. When this column was eluted at 45°C with a buffer (pH 2.91) containing 0.20 *M* sodium citrate, thiodiglycol (10 ml/l), isopropanol (10 ml/l), octanoic acid (0.1 ml/l) and phenol (1.0 ml/l) at a flow-rate of 30 ml/h, 4-hydroxyproline separated completely from aspartic acid and eluted from the column at 28.7 and 32.7 min (Fig. 3). Recoveries can be calculated relative to alanine, which elutes at 68.5 min. Although a large number of specific assays and chromatographic procedures for 4-hydroxyproline have appeared in the literature (reviewed in ref. 38), including a recent HPLC method [39], this single-column system, using pH 2.91 buffer, is a simple and

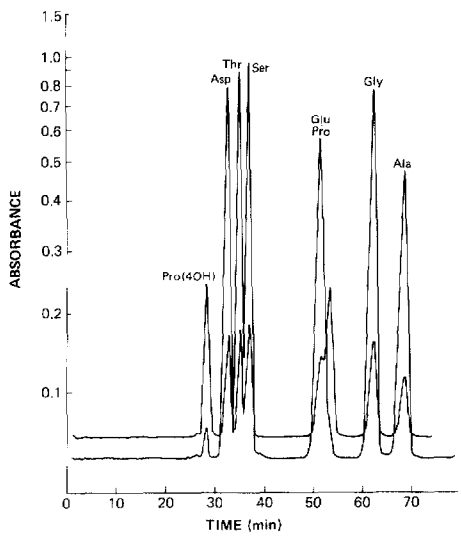

Fig. 3. Typical separation of 4-hydroxyproline [Pro(4OH)] from other amino acids on a 21 × 0.6 cm column of Dionex DC-6A resin. The upper curve shows the absorbance at 440 nm and the lower curve the absorbance at 570 nm. This column was eluted at 45°C with a buffer (pH 2.91) containing 0.20 *M* sodium citrate, thiodiglycol (10 ml/l), isopropanol (10 ml/l), octanoic acid (0.1 ml/l) and phenol (1.0 ml/l) at a flow-rate of 30 ml/h.

specific method for determining 4-hydroxyproline and 3-hydroxyproline not shown in the chromatogram of Fig. 3. Both the standard and accelerated systems have been applied for the analysis of tryptophan and 4-hydroxyproline in a variety of proteins and tissues, including foods and feedstuffs [28—30], and the results indicated that the reproducibility and accuracy of this procedure was 100 ± 3%.

A rapid analytical method has also been developed for determining all these unique basic amino acids at picomole levels in the minimum time using a 17.5 × 0.28 cm column on a fully automated amino acid analyser (equivalent to Beckman Model 121MB). High sensitivity and increased resolution were achieved, first, by selecting smaller-diameter microporous resins and a microbore (0.28 cm) column; second, by increasing the temperature of the ninhydrin reaction coil bath from 100 to 129 ± 0.1°C; and third, by amplifying the linear signal outputs of the photometer with the Varian Vista Model 402 chromatography data reduction system. Thus, by developing the 17.5 × 0.28 cm microcolumn of Dionex DC-5A resin with 0.21 *M* sodium citrate buffer (pH 5.125) at 52°C, and with an eluent linear flow velocity of 1.56 cm/min (5.75 ml/h) at 40 bar, excellent resolution of these basic amino acids was achieved. As may be seen in Fig. 4, the time required for a complete analysis by this system has been reduced from 4 [7] to 1.25 h, and since both tryptophan and the amino sugars elute early from the microcolumn, their analysis can be carried out in 48 and 65 min, respectively. The analysis time could be further reduced by increasing the flow-rate and temperature. However, the separation achieved by this system would be the method of choice for analysing complex protein or tissue hydrolysates, which on hydrolysis yield picomole levels of these amino acids. The microcolumn can be overloaded 100-

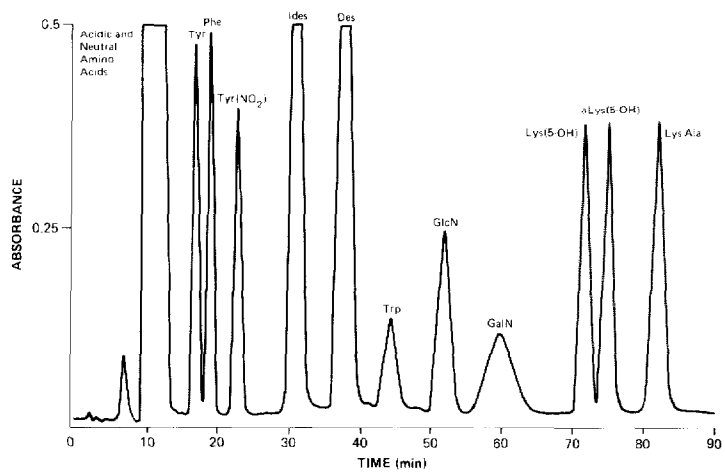

Fig. 4. Chromatographic separation of a synthetic mixture of eleven amino acids (0.5 nmol each) on an analytical 17.5 × 0.28 cm microcolumn of Dionex DC-5A resin. The microcolumn was operated at 52°C with 0.21 *M* citrate buffer (pH 5.125) containing 1% isopropanol. Buffer and ninhydrin flow-rates were 5.75 and 5.60 ml/h, respectively. The curve shows absorbance at 570 nm. Peaks: Tyr(NO_2) = 3-nitrotyrosine; Ides = isodesmosine; Des = desmosine; GlcN = glucosamine; GalN = galactosamine; Lys(5OH) = 5-hydroxylysine; aLys(5OH) = *allo*-5-hydroxylysine; LysAla = lysinoalanine.

to 500-fold (100—500 μg protein per 100 μl), provided the ninhydrin reagent is introduced into the buffer eluent stream after emergence of the acidic and neutral amino acids from the microcolumn.

The overall gain in sensitivity of this system, compared with the accelerated procedure (Fig. 2), was approximately 100-fold. A large increase in sensitivity resulted from the smaller bore and volume of the microcolumn using the 6-μm diameter Dionex type DC-5A spherical resin beads, which allowed the resin bed length to be reduced to 17.5 cm, while still providing excellent separation of these amino acids. Similar separations were obtained using a 20 × 0.28 cm microcolumn packed with either Dionex DC-4A or Beckman type W-3H resins, sized to 9.0 ± 0.5 μm; however, the sensitivity of these systems was lowered by a factor of ca. 2. The results shown in Fig. 4 are typical of the chromatographic separations obtained when a sample load of 500 pmol of each of the amino acid components of a synthetic calibration mixture were analysed by this system. When the absorbance range of the colorimeter control unit was set on the 0.5 scale for both the 570- and 440-nm channels, and the attenuation on the Vista 402 was set at 32, a sample load of 500 pmol of 3-nitrotyrosine gave an absorbance of ca. 0.4 (Fig. 4). Most of the other amino acids gave peaks that ranged in absorbance from 0.125 to 0.50, depending on their position on the chromatogram, except for desmosine and isodesmosine, which gave peak absorbances above the 0.5 range. The signal-to-noise ratio was 3.4 for the 570-nm channel and 5.6 for the 440-nm channel. The lower limit for detection by the system was ca. 10—20 pmol. Since only one buffer was used as the eluent, the buffer contaminants became an integral part of the baseline and did not interfere with any of the amino acids. It has thus been possible to achieve accurate quantitative determinations of these unusual amino acids at the 50—200 nmol levels, with a reproducibility of 100 ± 2.5%. This method has been applied successfully to the determination of these basic amino acids in a variety of proteins and tissues, including amyloid protein [40] and 10-formytetrahydrofolate dehydrogenase [41].

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