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HIGH-SENSITIVITY PHENYLTHIOHYDANTOIN AMINO ACID ANALYSIS USING CONVENTIONAL AND MICROBORE CHROMATOGRAPHY

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SUMMARY

Reversed-phase microbore high-performance liquid chromatography was investigated for high-sensitivity analysis of phenylthiohydantoin (PTH) amino acids. A mixed nitrile alkylsilane bonded phase was developed and ternary gradient elution conditions were devised for resolution of the common PTH amino acids. Elution conditions were developed with a conventional 150 × 4.6 mm I.D. column and transferred to a 150 × 1 mm I.D. microbore column. The performance of these columns was evaluated in terms of PTH amino acid resolution, enhanced sample detectability, and retention time precision. For this work a general purpose high-performance liquid chromatograph was modified to reduce extra column band broadening and a preformed gradient elution technique was developed to achieve rapid analysis times at microbore flow-rates. The microbore high-performance liquid chromatographic system is useful for high-sensitivity analysis of PTH amino acids in microsequencing applications.

INTRODUCTION

Trends in biomedical research have required the generation of protein sequence information from increasingly limited amounts of material. Recent advances in protein microsequencing technology have enabled acquisition of reliable sequence data from sub-nanomole quantities of polypeptides, and sequencing at the low picomole level is considered to be within the capability of existing microsequencing methods and instrumentation. It is anticipated that refinements in Edman-based approaches in the near future will permit sub-picomole sequencing of polypeptides eluted from two-dimensional gels [1].

The excellent resolution and reproducibility of reversed-phase chromatography, coupled with the high sensitivity of ultraviolet absorbance detectors have made high-performance liquid chromatography (HPLC) the method of choice for identification of the phenylthiohydantoin (PTH) amino acids generated by Edman degradation [2]. While the performance of existing HPLC

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instrumentation permits analysis of PTH amino acids (PTHAA) at the 5—10 pmol level, reduction of detection limits to subpicomole levels will require improvements in detection methods. Sequence information has been obtained at the 100-fmol level using radioactively labelled peptides [1]. Such improvements could encompass the use of modified phenylisothiocyanate (PITC) reagents with enhanced detectability [3] or the use of laser-based optical detectors with enhanced sensitivity [4]. An alternative approach is the use of microbore HPLC columns to achieve increased solute detectability by reducing peak dilution. Since peak volume is proportional to column cross-sectional area, reduction of column internal diameter from 4.6 mm to 1 mm can produce up to a twenty-fold increase in peak concentration for the same sample mass.

Microbore HPLC has several advantages compared to other means of enhancing detectability. First, many commercial HPLC systems are compatible with microbore columns or can be modified with relative ease for microbore chromatography. Secondly, microbore columns are typically packed with 4—10 μm microparticulate silica-based bonded-phase materials and therefore exhibit selectivity and efficiency comparable to that of the conventional 4 mm and 4.6 mm I.D. reversed-phase columns currently used for PTH amino acid analysis. Thirdly, microbore chromatography can be used as a complementary means of sensitivity enhancement in conjunction with improved detector design and derivatization chemistries.

We have investigated the utility of microbore HPLC for high-sensitivty analysis of PTH amino acids in microsequencing. This report will cover three aspects of this investigation: (a) development of a specific reversed-phase support and elution conditions for rapid resolution of the common PTH amino acids, (b) configuration of a general-purpose high-performance liquid chromatograph for operation with 1 mm I.D. columns, and (c) application of a preformed ternary gradient elution technique permitting reproducible chromatography of PTH amino acids at microbore flow-rates.

MATERIALS AND METHODS

Instrumentation included a Varian 5500 liquid chromatograph equipped with a UV-200 detector (4.5 μl flow cell, 4-mm path length) or, as noted, an optional 0.5-μl (2-mm pathlength) flow cell. A Rheodyne Model 7125 (10-μl loop) or automated 7410 (1-μl loop) injection valve was used. Data acquisition, reduction and automatic sampling were accomplished using a Varian Vista CDS 402 data system and 8085 autosampler.

Standards were prepared in methanol, stored at —20° C, and diluted daily to appropriate concentrations with the starting mobile phase.

Individual PTH amino acid standards were obtained from Sigma (St. Louis, MO, U.S.A.) and a premixed PTH standard was obtained from Pierce (Rockford, IL, U.S.A.).

Mobile phases for HPLC of PTH amino acids included methanol and acetonitrile (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). Buffers were prepared using HPLC-grade sodium acetate and potassium dihydrogen phosphate (Fisher Scientific, Pittsburgh, PA, U.S.A.). HPLC-grade water was generated using Hydro Service's (Varian, Sunnyvale, CA, U.S.A.) water purification system.

The CN (150 × 4 mm, particle size 5 μm) and C_{18} (150 × 4.6 mm, particle size 5 μm) columns were custom synthesized and the PTHAA column, a mixed nitrile and alkyl silane bonded phase (150 × 4.6 mm and 150 × 1 mm, particle size 4 μm) was purchased from Varian.

A 2 cm × 2 mm guard column (Upchurch Scientific, Oak Harbor, WA, U.S.A.) was packed with Vydac 40-μm pellicular C_{18} packing. Addition of the guard column had little effect on overall resolution using the 4.6 mm I.D. column. No guard column was used with the 1.0 mm I.D. column.

RESULTS AND DISCUSSION

Stationary phase optimizations

The first step in developing any separation is selection of the proper column. Previous work in our laboratory as well as other published material showed that either a CN (nitrile) column or C_{18} column could be used for PTH amino acid separations. Inspection of the literature reveals many methodologies using both types of column [2, 5—10]. Although columns from various manufacturers differ widely, most, including the MicroPak CN and MicroPak C_{18}, offer better selectivity for different regions of the PTH separation. An example of this can be seen in Fig. 1. Upon inspection, it is apparent the CN column is a better column for resolving the later eluting peaks Met, Ile, and Tyr, but a C_{18} column can best resolve the early eluting components between Cys and Met. Clearly, a stationary phase combining the best selectivity of these two phases would be ideal for PTH amino acid separations.

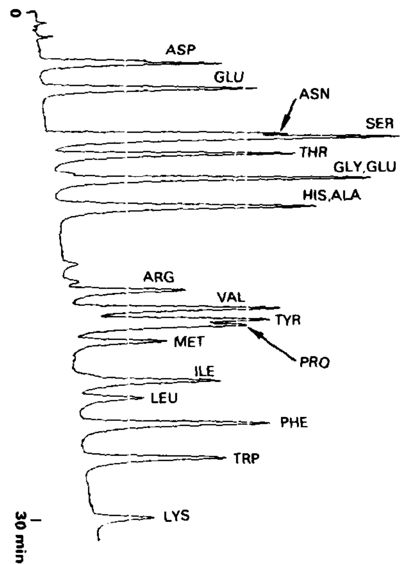
Experiments using a C_{18} column in series with a CN column were not successful and therefore a stationary phase was synthesized that had both CN and alkyl character. The relative molar ratios of CN and alkyl carbon were adjusted in order to obtain a column with maximum selectivity for the separation of PTH amino acids, herein referred to as a PTHAA column.

Mobile phase optimization of PTH separation

The mobile phase used with the PTHAA column was adjusted empirically to improve selectivity. Experiments with pH and the ionic strength of the buffer showed that the PTH derivatives of aspartic acid, cysteic acid, glutamic acid, histidine and arginine were the most sensitive to changes in these mobile phase parameters. Increase in ionic strength will decrease the retention time of His and Arg. An increase in pH in the range 2.0—6.5 will decrease the retention of the acidic PTH amino acids Asp, Glu and Cys and increase the retention times of Arg and His. Increasing the gradient slope will, paradoxically, increase the resolution of Pro, Val, Tyr and Met.

Simple linear gradients from buffer—methanol (90:10) to 60% methanol were compared to gradients in which acetonitrile had been substituted for methanol. Such experiments showed that use of methanol provided maximum selectivity for separation of the early eluting PTH amino acids between Asp and Arg but resulted in poor resolution of the more hydrophobic PTH amino acids between Tyr and Lys. Using acetonitrile had the opposite effect. In such a circumstance ternary gradients can be exploited to maximize PTH separation. Fig. 2A is a separation using a ternary gradient such that methanol is the principal strong solvent in the beginning of the chromatogram and acetonitrile

A



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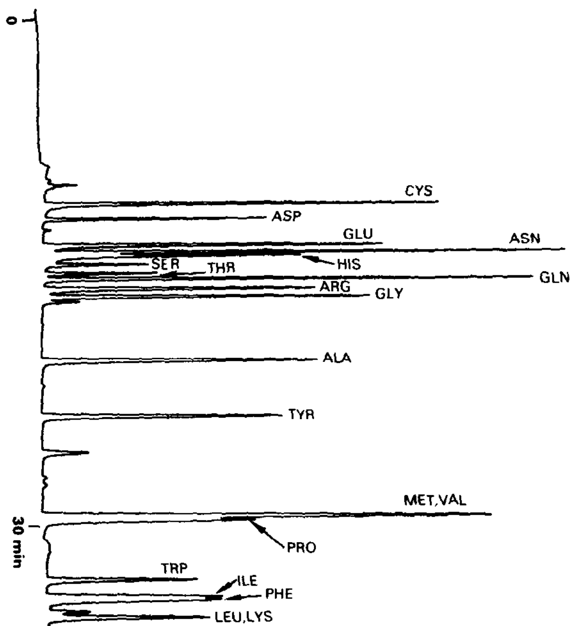

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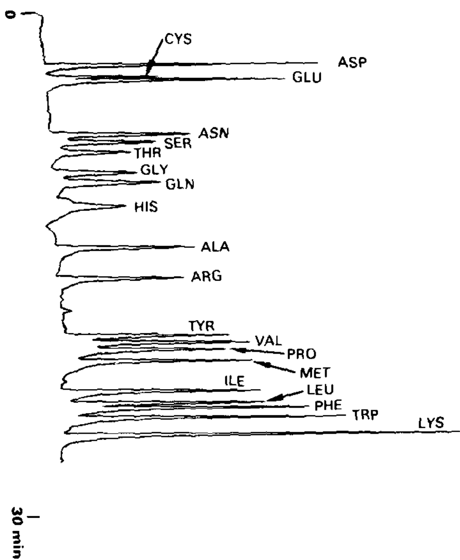


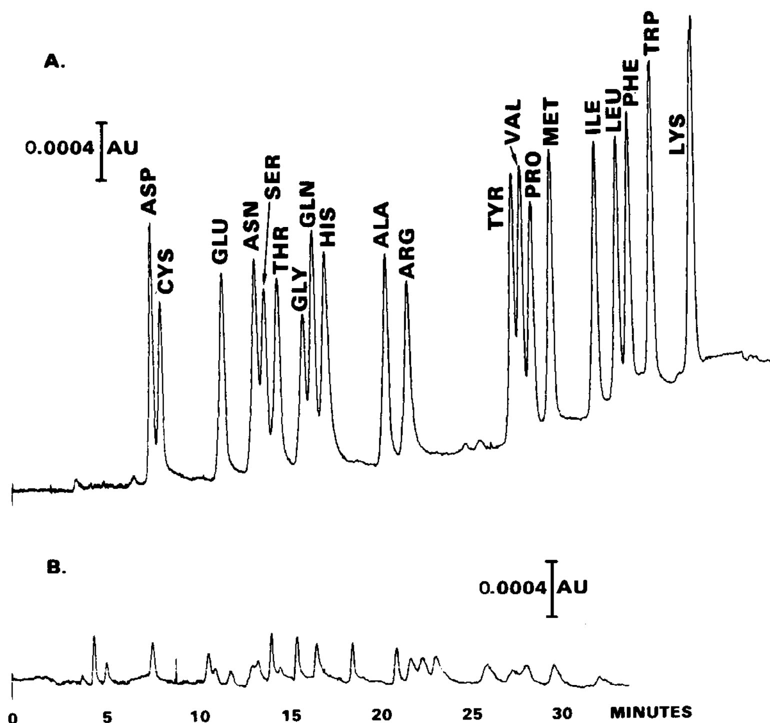


Fig. 2. Comparison of a 10-pmol injection of PTH amino acid using (A) a 15 cm × 1 mm I.D. Microbore-1 PTHAA-4 column and (B) a 15 cm × 4.6 mm I.D. MicroPak PTHAA-4 column. (A) Gradient conditions for the microbore column can be found in the text and in the legend to Fig. 3B. Solvent A: 0.01 *M* sodium acetate, pH 5.0; solvent B: methanol; solvent C: acetonitrile. Temperature 30°C; flow-rate 50 μl/min; 1-μl injection; detection at 270 nm with a UV-200 detector and 0.5-μl flow cell. (B) Solvent A: 0.01 *M* potassium dihydrogen phosphate, pH 5.0; solvent B: methanol; solvent C: acetonitrile. Gradient conditions for the conventional column: t = 0 min, A = 92%, C = 8%; t = 4 min, A = 85%, B = 15%; t = 8.0 min, A = 85%, B = 10%, C = 5%; t = 8.1 min, A = 85%, C = 15%; t = 12.0 min, A = 70%, C = 30%; t = 30 min, A = 40%, B = 60%. Temperature 30°C; detection at 270 nm with a UV-200 detector and 4.5-μl flow cell. Same attenuation on both columns.

Fig. 1. PTH separation on three different columns. (A) Column type CN, 5 μm particle size, 15 cm × 4.0 mm I.D.; flow-rate 1.3 ml/min; temperature 30°C. Solvent A: 0.05 *M* potassium dihydrogen phosphate, pH 6.5; solvent B: acetonitrile. Solvent program: t = 0 min, A = 95%; t = 10 min, A = 75%; t = 27 min, A = 40%. (B) Column type C_{18}, 5 μm particle size, 15 cm × 4.6 mm I.D.; flow-rate 1.0 ml/min. Solvent A: 0.05 *M* potassium dihydrogen phosphate, pH 5.0; solvent B: acetonitrile. Solvent program: t = 0 min, A = 95%; t = 10 min, A = 75%; t = 27 min, A = 60%; t = 37 min, A = 50%. (C) Column type PTHAA, 4 μm particle size, 15 cm × 4.6 mm I.D.; flow-rate 1.3 ml/min; temperature 30°C. Solvent A: 0.05 *M* sodium acetate, pH 6.0; solvent B: methanol; solvent C: acetonitrile. Solvent program: t = 0 min, A = 85%, B = 10%, C = 5%; t = 8.0 min, A = 85%, B = 10%, C = 5%; t = 8.1 min, A = 85%, C = 15%; t = 30 min, A = 40%, C = 60%. Injection of 10 μl on all columns.

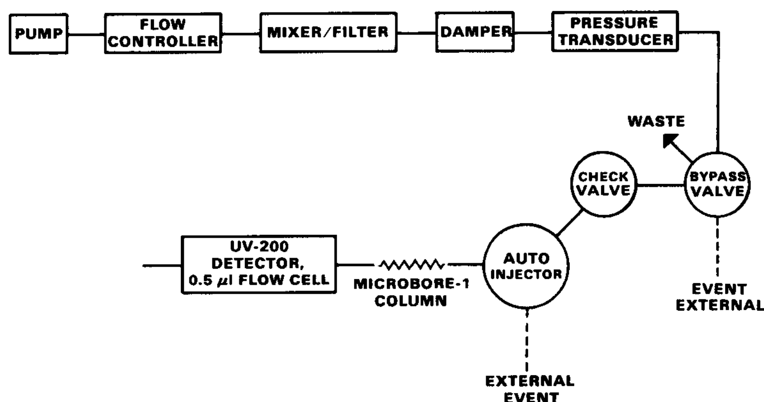
at the end. Varying the relative ratios of methanol/acetonitrile allows one to optimize resolution and compensate for changes in selectivity that may occur over the lifetime of the column. Increases in ionic strength will decrease the retention time of His and Arg. Decreasing the starting solvent strength will increase the resolution of the early eluting PTHs between Asp and Arg.

The column temperature was thermostated at 30°C to improve retention time reproducibility. Experiments at higher temperatures (40—60°C) did not improve the resolution of the solutes.

Microbore chromatography

Once the mobile phase conditions had been selected on a conventional

A.



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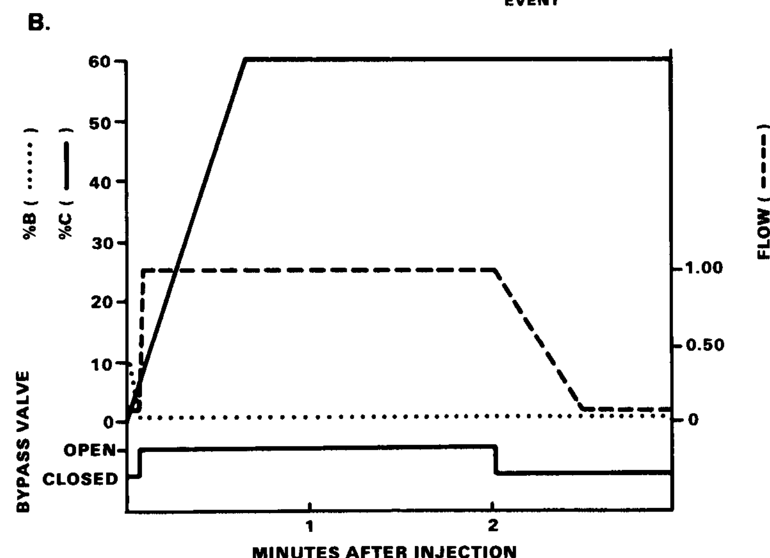

Fig. 3. (A) Scheme of high-performance liquid chromatograph components for microbore chromatography; external events are relays controlled by the instrument microprocessor. (B) Timing diagram used in preforming a microbore gradient. Injection was made at time 0. Solvent A = 0.01 *M* sodium acetate, pH = 5.0; solvent B = methanol; solvent C = acetonitrile. Flow-rate in ml/min. Bypass valve opened 0.1 min after injection, shown here slightly later for clarity.

15 cm × 4.6 mm I.D. column, it was necessary to devise a method to generate a mobile phase gradient using the 1 mm I.D. microbore column at a flow-rate of 50 μl/ml. The LC 5500 chromatograph is capable of forming gradients at microbore flow-rates since solvent proportioning is accomplished during the fill stroke of the single-piston pump and is therefore independent of flow-rate. However, the volume between the pump and injector is about 2 ml so the time delay between gradient formation at the pump delivery to the column top is excessive at 50 μl/min. This time delay can be overcome by using a preformed gradient technique. A variation on a technique outlined by Katz and Scott was explored [11]. Fig. 3 represents a diagram of the system and a listing of the timed events required to carry out such a preformed gradient for PTH amino acid separation. Valve switching is controlled by the instrument microprocessor to insure reproducibility. The injection is made at time 0. At 0.01 min an automatic bypass valve is opened, and the microbore gradient is formed at 1 ml/min over a 0.60-min period. This gradient is then pumped through the hydraulic volume in the chromatograph, and at 2.0 min the bypass valve is closed. At this time the system is pressurized as the flow-rate is gradually reduced over 0.5 min to 50 μl/min. The time at which the bypass valve closes determines when the gradient reaches the column head and may be varied in order to control the initial isocratic portion of the mobile phase program. A check valve is placed in the system between the bypass valve and the injector to minimize rapid column pressure drops when the bypass valve is opened. Gradient profiles were calculated and checked by doping the final solvent with 0.1% acetone. Preformed microbore gradient profiles generated upon running undoped to doped solvents were compared to direct gradients and adjusted to achieve congruency with the direct-gradient slope.

Once the preformed gradient profile has been programmed into the LC 5500 chromatograph, operation is no different than in a conventional mode, i.e. the injector is turned to the inject position and the program started.

Fig. 2 shows a comparison of 10-pmol injections on a conventional 4.6 mm I.D. column versus a 1.0 mm I.D. column. Note the selectivity in both systems is very similar, but the peak signals with microbore are greater.

Sensitivity

The primary reason for investigating microbore chromatography of PTH amino acids was to determine the actual increase in sensitivity measured as signal-to-noise ratio (S/N) gained by reducing the column internal diameter from 4.6 to 1.0 mm.

Detection levels and sensitivity in chromatography are related to a large number of variables including: injection volume, column diameter, path length (UV detectors), noise level, wavelength, peak volume, k', column efficiency and extra column band broadening.

Extra column band broadening can significantly decrease the observed column efficiency resulting in decreased S/N. Reducing the flow-cell volumes without increasing noise is a necessary prerequisite when operating with 1 mm I.D. columns. Substituting a 1.0 mm I.D. column for a 4.6 mm I.D. column without consideration of extra column effects can lead to a decrease in sensitivity and severe losses in resolution. Methods for measuring and quantifying such effects have been outlined by Kok et al. [12].

The major features of the microbore HPLC system used in this study include (1) a 1-μl injector (2) 0.13 mm I.D. connecting tubing and (3) a 0.5-μl flow cell.

For the system tested, the theoretical increase in S/N should be about 10.5 × [i.e. (4.6 mm I.D./1.0 mm I.D.)^2 divided by 2 to account for the different flow cell path lengths of the 0.5- and 4.5-μl flow cells]. This factor assumes the noise level is the same for both flow cells. This was verified experimentally. In a parallel experiment, an isocratic elution of PTH alanine was performed using the 4.5-μl, 4.0-mm path length, flow cell and a 1-μl injector on both 15 cm × 4.6 mm I.D. and 15 cm × 1.0 mm I.D. columns (Fig. 4). The flow-rate was adjusted to give the same retention time for PTH alanine on both columns. This experiment is in agreement with the gradient results in that a 8 × improvement in S/N is observed when a correction for differences in path length is made.

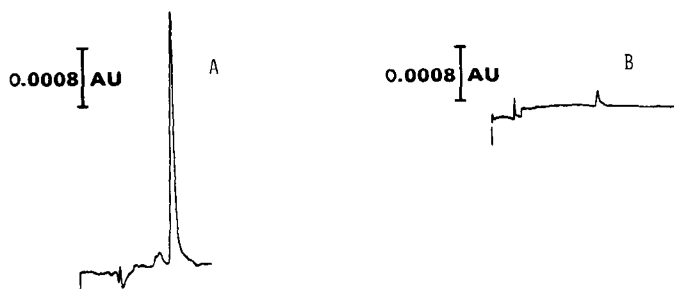

Fig. 4. Isocratic elution of PTH alanine using a 4.5-μl flow cell and 1-μl injection for both columns. All conditions except flow-rate and column diameter were identical, 2.4 pmol per injection. (A) Microbore column, 15 cm × 1.0 mm I.D., flow-rate 0.06 ml/min, S/N = 82. (B) Conventional column, 15 cm × 4.6 mm I.D., flow-rate 1.0 ml/min, S/N = 5.

Precision

Precision data for both microbore and conventional gradient systems are exhibited in Table I. As expected, the relative standard deviations are higher for preformed gradients but nevertheless adequate for PTH amino acid identification. The PTH amino acids were chosen to represent acidic, basic and neutral PTH derivatives.

TABLE I

RETENTION TIME PRECISION IN PTH AMINO ACID ANALYSIS, NINE CONSECUTIVE RUNS

Retention time (R.T.) and standard deviation (σ) in min, and relative standard deviation (R.S.D.) in percent.

Column (PTHAA)	Glu			Arg			Lys		
	R.T.	σ	R.S.D.	R.T.	σ	R.S.D.	R.T.	σ	R.S.D.
Conventional, 4.6 mm I.D.	3.81	0.02	0.5	15.27	0.06	0.4	25.70	0.03	0.1
Microbore, 1 mm I.D.	9.66	0.11	1.1	17.82	0.11	0.6	32.58	0.38	1.1

CONCLUSIONS

The use of a mixed stationary phase offers high selectivity for separation of PTH amino acids. Microbore HPLC on 1.0 mm I.D. columns offers a significant

improvement in sensitivity. This places the minimum detectable quantity of PTH derivatives in the range 0.5—1.0 pmol.

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