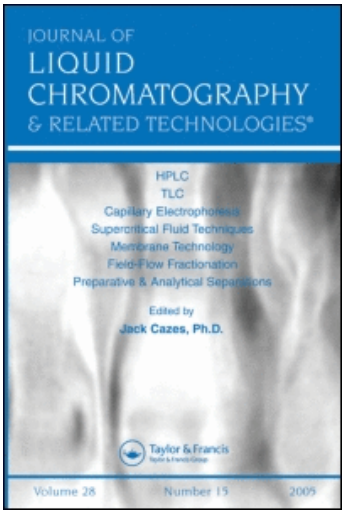

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C. B'Hymer[a]
[a] Department of Chemistry, University of Cincinnati, Cincinnati, OH, U.S.A.

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EVALUATION AND OPTIMIZATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC CONDITIONS FOR OPTICAL PURITY ANALYSIS OF AMINO ACID RESIDUES FROM A SYNTHETIC DECAPEPTIDE USING MARFEY'S REAGENT

C. B'Hymer

Department of Chemistry, University of Cincinnati,
Mail Location 0172, Cincinnati, OH 45221-0172, USA

ABSTRACT

An optimized liquid chromatographic system was developed to separate a mixture of Marfey's amino acid derivatives (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, FDAA). Alanine, glutamic acid, isoleucine, proline, and tyrosine were the five natural amino acid residues from the synthetic decapeptide, Suc-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-Glu-OH, which was analyzed for optical purity as part of this study. Excellent chromatographic resolution of the five pairs of natural amino acid derivatives, including D-alanine and D-proline, within a 70 minute gradient elution using an ODS (C-18) column, was obtained using acetonitrile as the organic modifier and sodium acetate buffer. The effects of the mobile phase pH with respect to peak retention factor (k) and separation factor (α) were studied in this chromatographic system.

INTRODUCTION

The importance of stereoisomerism with respect to biological activity has been recognized since the original discovery of optical isomerism of tartaric acid by Pasteur in 1848. The physiological environment within living organisms is chiral, and biological activity of the enantiomeric forms of molecules is different. The separation of racemic mixtures into its steroisomer components is an analytical challenge and of intense interest. The development of chromatographic methods to perform separation of enantiomers has been an important area of study within the pharmaceutical industry for the last two decades.

Two main strategies have evolved over the past years to separate enantiomers, each with their separate advantages and disadvantages. One strategy involves the reaction of the racemic analytes with an optically active reagent for a pair of diastereomers(1), which have different physical properties and can be separated by conventional chromatographic procedures. The second strategy, which has gained in popularity in recent years, is to use chiral chromatography. The latter strategy utilizes a chiral selector in either the stationary phase or the mobile phase. In general, this direct approach of chiral chromatography offers less chance of enatiomer selectivity or bias, but usually is more costly and complicated chromatographically. In this paper, a low cost and effective derivatization approach using Marfey's reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA), will be discussed.

The use of Marfey's reagent for derivatization of amino acids in optical purity analysis was first reported by Marfey in 1984.(2) FDAA has a chiral center in its alanine group, the L form, and when it reacts with amino acids, it produces the analogous diastereomers (see Figure 1). These diastereomers can be separated using reversed phase HPLC. This chromatographic separation has been attributed to the formation of an intramolecular hydrogen bond between the carboxy and carboxyamide group in the L-L diastereomer and the non-formation of this hydrogen bond in the D-L diastereomer.(2-3)

Marfey's reagent has been used extensively for the determination of optical purity for both amino acids and small peptides.(1,3-7) Acid hydrolysis of peptides and the use of Marfey's reagent has been demonstrated to generate accurate results with little racemization of amino acid isomer residues.(7) Other uses of Marfey's reagent has included the optical purity analysis of synthetic amino acid analogs(8,9) and drug compounds containing amine functional groups.(10) Montes *et al.*(11) has successfully used Marfey's reagent for optical purity analysis of seleno amino acids contained in selenium inoculated yeast and synthetic selenomethionine in pharmaceutical preparations.

Alanine, glutamic acid, isoleucine, proline, and tyrosine were the five natural amino acid residues of interest from the decapeptide analyzed. Problems occurred when attempting to separate all of these FDAA derivatized amino acid

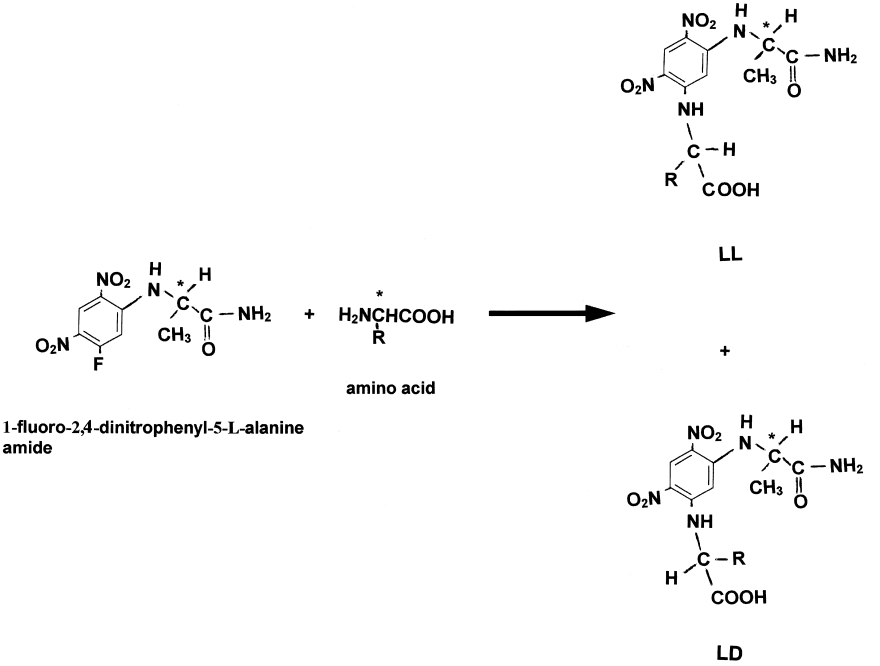

Figure 1. Reaction of DL-amino acids with Marfey's reagent.

enatiomers in a single chromatographic run; specifically, the D-alanine and D-proline derivative peaks were difficult to resolve from one another. Also, adding to the chromatographic separation complexity is the derivatization products of tyrosine. Marfey's reagent, FDAA, reacts with the hydroxyl group of tyrosine,(3) thus, mono and bis tyrosine products will appear as separate peaks chromatographically. Another aim of this work was to observe any changes in bis tyrosine derivative production by adjusting the basic conditions of the Marfey's derivatization process. Additionally, the optimized chromatographic conditions and procedure developed required optical purity quantitation levels of 0.1% D amino acids to the natural L amino acids observed. The synthetic decapeptide, Suc-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-Glu-OH, contains a succinyl capped N-terminus and the synthetic cyclohexylalanine at position nine. This decapeptide was under study for medicinal properties and optical purity determination of the amino acids was essential.

This paper describes optimized HPLC conditions, which are capable of separating the FDAA derivatives of alanine, glutamic acid, isoleucine, proline, tyrosine, and cyclohexylalanine using a single chromatographic run. It also

describes the effects of mobile phase pH on the retention factor (capacity factor), k, and separation factor (selectivity), α, of the natural amino acid derivatives.

EXPERIMENTAL

Reagents

Marfey's reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA), was purchased from Pierce Chemical Company (Rockford, Illinois, USA). The standard L and D amino acids of alanine, glutamic acid, isoleucine, proline, and tyrosine were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). High purity HPLC water was provided by a Barnstead (Boston, Massachusetts, USA) NANOpure system, followed with an ultraviolet radiation treatment by a Barnstead OGANICpure system. HPLC grade acetonitrile was purchased from Burdick & Jackson (Muskegon, Michigan, USA). The synthetic decapeptide was from an "in-house" source. All other reagents were commercial reagent grade.

Chromatographic Conditions and Apparatus

A Spectra-Physics (San Jose, California, USA) Model SP8800 liquid chromatograph equipped with a Rheodyne (Coatati, California, USA) Model 7010 injector valve and a Chromanetics (Vineland, New Jersey, USA) 5 μm Spherisorb ODS analytical column (250 X 4.6 mm ID) were used. UV detection at 340 nm was accomplished with an Applied Biosystems (PE Biosystem, Norwalk, Connecticut, USA) Model 757 detector. The mobile phase flow rate was maintained at 1.7 mL/min. Mobile phase A consisted of acetonitrile/water (10:90, v/v) and 0.04 M in sodium acetate concentration (apparent pH adjusted to 5.3). Mobile phase B consisted of acetonitrile/water (50:50, v/v) and was also 0.04 M in sodium acetate buffer (apparent pH adjusted to 5.3).

The gradient program utilized three linear steps, 0 to 20 % B in thirty minutes for the first step, 20 to 75% B in the next thirty minutes for the second step, and 75 to 100% B in 10 minutes for the last step. A ten minute hold at 100% B was used at the end of the gradient program before returning the initial conditions. Injection size was 20 μL of the sample solutions.

FDAA Derivatization of Amino Acids

The derivatization procedure used was adapted from Marfey's original procedure.(2) Solutions of the individual and a combined mixture of L and D ena-

tiomers of alanine, glutamic acid, isoleucine, proline, and tyrosine were used. A 150 µL volume of an aqueous solution containing 30 micromoles amino acid and 0.3 M sodium bicarbonate was treated with 200 µL of 1% (w/v) FDAA in acetone. The solution was reacted for two hours at 40°C, then 20 µL of 2M hydrochloric acid were added to stop the reaction. A 1:200 dilution was made in mobile phase A to obtain the chromatographic sample solution.

Mono and Bis Derivatives of Tyrosine

Three sets of reaction conditions were used to study the formation of mono and bis derivatives of tyrosine. In the first experiment, the molar ratio of FDAA to tyrosine was varied. In the second experiment, the length of reaction time between FDAA and tyrosine was varied. In the last experiment, varying amounts of 0.3M sodium hydroxide were used in place of sodium bicarbonate.

Analysis of Synthetic Decapeptide

A sample of decapeptide (approximately 40 mg) equivalent to 300 micromoles in total amino acids was dissolved in 1.0 mL of 6 M hydrochloric acid and hydrolyzed for six hours at 110°C. The solution was cooled, and a 0.5 mL portion was neutralized with 2.5 mL of 1 M sodium bicarbonate. A 100 µL portion of this solution was treated with 50 µL of 1 M sodium bicarbonate and reacted with 200 µL of 1% (w/v) FDAA/acetone reagent for two hours at 40°C. The reaction solution was treated with 20 µL of 2 M hydrochloric acid and then diluted to 5.0 mL with mobile phase A to make the chromatographic sample solution.

Effect of Mobile Phase pH

The same chromatographic conditions listed previously were used with the exception of the apparent pH of the mobile phase. The FDAA amino acid derivatives were chromatographed using 0.04 M sodium acetate mobile phases with a pH range between 3.5 to 6.0. The apparent pH of the mobile phase was adjusted by the addition of 10 M sodium hydroxide solution.

Calculations

The retention factors (capacity factors), k, were calculated by the standard formula: $k = (t_r - t_0)/t_0$, where t_r is the retention time of the analyte and t_0 is the non-retained or dead volume time.(12-14) The separation factor (selectiv-

ity), α, was calculated by the standard formula: $\alpha = k_2/k_1$, which is the ratio of the retention factors for the amino acid derivative L, D pairs in this analysis.(12-14)

DISCUSSION

Using the optimized HPLC conditions described in this work [the three step gradient at pH 5.3 buffering], five natural amino acid enatiomer pairs of FDAA derivatives can be easily resolved. The separation of an enatiomeric mixture of alanine, glutamic acid, isoleucine, proline and tyrosine is shown in Figure 2. Marfey's reagent derivatives typically have the L amino acid derivative elute before the D isomer.(4) As mentioned in the introduction, tyrosine always forms mono and bis

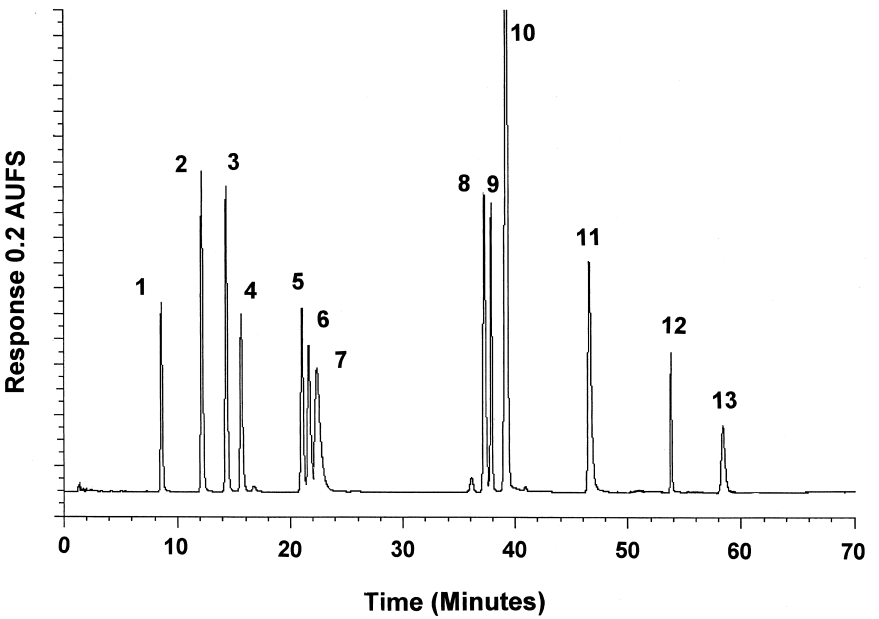

Figure 2. Chromatogram of a mixture of five pairs of natural amino acid Marfey's derivatives. Peaks are as follows: 1 = L-glutamic acid , 2 = D-glutamic acid, 3 = L-alanine, 4 = L-proline, 5 = L-tyrosine (mono derivative), 6 = D-proline, 7 = D-alanine, 8 = L-isoleucine, 9 = D-tyrosine (mono derivative), 10 = excess hyrdrolyzed Marfey's reagent, 11 = D-isoleucine, 12 = L-tyrosine (bis derivative), and 13 = D-tyrosine (bis derivative). Each amino acid is at a concentration of approximately 0.4 mM. The two small peaks at retention times 36 and 41 minutes are related to excess Marfey's reagent.

derivative peaks. Also, the excess Marfey's reagent is hydrolyzed and produces a peak at retention time 39 minutes by these chromatographic conditions.

The mono L-tyrosine, D-proline, and D-alanine derivative peaks eluted very near each other as did L-isoleucine and mono D-tyrosine derivative peaks. At mobile phase pH 5.3, resolution of 1.0 to 1.2 were constantly obtained between these adjacent peaks. This was the best that could be obtained with this chromatographic system, and it was adequate for peptide residue analysis. (Lower concentrations of the D-proline and D-isoleucine derivatives gave better resolution than shown in Figure 2.)

A chromatogram of derivatized residue from the synthetic decapeptide is shown in Figure 3. The cyclohexylalanine derivative peak is resolved from the other amino acid derivative peaks; in Figure 3 only the L form is present in this particular sample and elutes at a retention time of 51 minutes. Some D-glutamic acid is in the synthetic process of the decapeptide and is clearly shown in the chromatogram at the expected level.

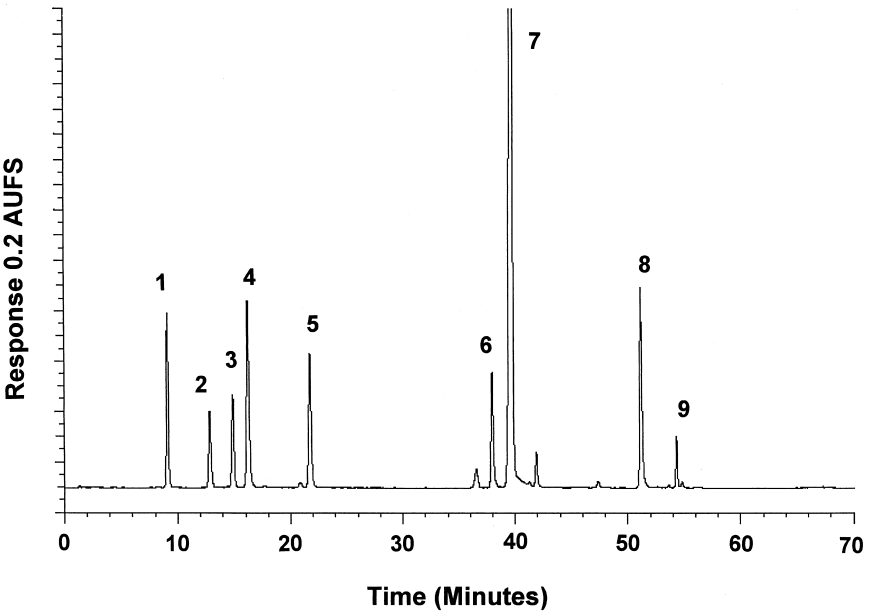

Figure 3. Chromatogram of the hydrolyzed residues of synthetic decapeptide. Peaks are as follows: 1 = L-glutamic acid , 2 = D-glutamic acid, 3 = L-alanine, 4 = L-proline, 5 = L-tyrosine (mono derivative), 6 = L-isoleucine, 7 = excess hyrdrolyzed Marfey's reagent, 8 = L-cyclo-hexylalanine, and 10 = L-tyrosine (bis derivative). The two small peaks at retention times 37 and 42 minutes are related to excess Marfey's reagent.

The pH of the mobile phase was found to have significant impact upon the retention factor (k) and separation factor (α) of the FDAA amino acid derivatives, and the data obtained at various pH levels are summarized in Table 1. Using the same gradient and organic modifier content, optimal separation for the enatiomer studied was obtained between pH 4.0 and 5.5. The separation factor of the bis derivatives of D- and L- tyrosine was virtually unchanged over the tested pH range; the separation factor remained between 1.07 and 1.10. Likewise, the retention factors of the bis derivatives of D- and L-tyrosine were also little changed by pH. However, the retention factors of the derivatives of D- and L-glutamic acid were the most affected by pH changes. In general, retention factors of FDAA amino acid derivatives decreased as pH was increased.

The reaction condition study of the mono/bis tyrosine derivatives showed that the mono derivative could not be entirely eliminated using the aqueous sodium bicarbonate or sodium hydroxide conditions. Increasing the reaction

Table 1. Retention (k) and Separation (α) Factors of Marfey's (FDAA) Derivatives at Different Mobile Phase pH Levels

Amino Acid Derivative		Retention Factor (k) and Separation Factor (α) at pH						
		3.5	4.0	4.5	5.0	5.3	5.5	6.0
Alanine	L retention factor	18.8	13.7	11.7	11.6	12.4	12.1	10.5
	D retention factor	33.8*	21.8	19.0	18.7	20.7	19.4	17.4
	Separation factor	1.80	1.59	1.62	1.61	1.67	1.60	1.66
Glutamic Acid	L retention factor	17.2	10.2	9.3	8.0	7.5	6.0	3.8
	D retention factor	26.0	14.9	13.9	11.9	11.1	9.6	5.2
	Separation factor	1.51	1.46	1.49	1.49	1.48	1.45	1.36
Isoleucine	L retention factor	44.9	36.4	34.3	32.8	33.4	32.5	29.9
	D retention factor	50.6	47.1	43.9	41.8	42.2	41.0	38.8
	Separation factor	1.13	1.30	1.28	1.27	1.26	1.26	1.30
Proline	L retention factor	25.0	14.5	13.1	12.8	13.6	13.2	11.5
	D retention factor	33.4*	21.1	18.5	18.2	19.2	18.6	16.7
	Separation factor	1.33	1.46	1.42	1.42	1.41	1.41	1.46
Tyrosine	L retention factor	34.7	23.8	18.5	18.2	18.6	18.3	16.0
(mono)	D retention factor	40.0	33.2	33.9*	33.4	33.8	33.6	31.8
	Separation factor	1.15	1.39	1.83	1.83	1.82	1.83	1.99
Tyrosine	L retention factor	53.8	48.9	47.8	47.9	48.4	48.3	47.7
(bis)	D retention factor	56.8	53.3	51.3	51.9	53.0	53.0	52.1
	Separation factor	1.07	1.09	1.07	1.08	1.10	1.10	1.09

*Note: The amino acid derivative peak co-eluted with the excess Marfey's reagent peak in the chromatogram at this pH.

time to at least two hours, increasing the molar ration of FDAA reagent to tyrosine, and using a reaction solution of both sodium hydroxide and sodium bicarbonate instead of sodium bicarbonate alone were found to increase the yield of the bis tyrosine derivative.

Other interesting findings during this study included the stability of the derivatized amino acids in solution. The amino acid FDAA derivatives were light sensitive; one day exposure to sunlight was found to cause significant degradation of the sample solutions. The sample solutions were stable for at least five days when protected from light. Light sensitivity of Marfey's derivative solutions has not been extensively discussed in any of the work published to date. Chromatographic findings early in this work included the fact that acetonitrile was a better organic modifier in the mobile phase than methanol. Acetonitrile gave better resolution and peak efficiencies over chromatographic gradient systems using methanol based mobile phases. Also, the organic content of the chromatographic sample solution must be as close to that of mobile phase A to avoid peak broadening, distortion, and splitting for L, D-alanine, L, D-glutamic acid, and L-proline derivative peaks in the optimized chromatographic system finally adopted.

The gradient separation demonstrated in this paper has been evaluated with other Marfey's derivative amino acids and has been found to separate in excess of thirty derivatives. This optimized gradient procedure at pH 5.3 buffer has been found to work well for the amino acid residues in the synthetic decapeptide used here and is certainly applicable to other small peptides. The sensitivity of this method was found to be adequate to detect 0.1% level of D amino acids spikes in L amino acid standards at the concentrations used in this study. Several batches of the synthetic decapeptide have been evaluated by the developed procedure, and the procedure appears to give reasonable optical purity estimate of the amino acid residues.

CONCLUSIONS

The Marfey's reagent derivatization procedure and these optimized chromatographic gradient conditions discussed here have been used to successfully separate and resolve the five amino acid isomer pairs of alanine, glutamic acid, isoleucine, proline, and tyrosine in a single chromatogram. This system was applicable in determining the optical purity of a synthetic decapeptide containing these amino acids. Mobile phase with an apparent pH of 5.3 gave the best separation for all the derivatives studied to separate them from one another, as well as, the excess hydrolyzed Marfey's reagent. This chromatographic system should be useful in the optical purity analysis of amino acid residues of similar small peptides.

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