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Journal of Chromatography A, 816 (1998) 59–64

JOURNAL OF CHROMATOGRAPHY A

Short communication

Determination of the chirality of cysteines in somatostatin analogs

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Abstract

A procedure to determine the chiral composition of cysteine in somatostatin analogs using C_{18} reversed-phase high-performance liquid chromatography is presented. As losses of cysteine are typically high during acid hydrolysis, cysteine residues were first protected by derivatization with 4-vinyl pyridine. The intact (chiral) liberated *S*-pyridylethylcysteine residues were identified by derivatization with Marfey's reagent. In the case of peptides containing bridged cysteines, a initial reduction step was required. Tris-(2-carboxyethyl) phosphine was used as a reducing agent. The procedure is fast and sensitive. No measurable hydrolytic racemization of the pyridylethyl cysteine was observed. Baseline resolution of the derivatized enantiomers was obtained with reversed-phase liquid chromatography.

Keywords: Enantiomer separation; Derivatization, LC; Cysteines; Somatostatin; Amino acids; Marfey's reagent

1. Introduction

The chiral purity of an amino acid in a peptide is usually determined by acid hydrolysis of the peptide and resolution of the amino acid using either direct or indirect chromatographic mixtures [1]. Cysteine, however, has historically posed a considerable challenge in this regard. Cysteine's highly reactive sulfhydryl group is prone to oxidation, elimination and modification by electrophilic compounds. Furthermore, as indicated by Woiwode et al. [2] peptides containing cysteine epimerize during hydrolysis at a rate which appears to be sequence dependent. The sulfhydryl group forms thiazoline adduct with *N*-adjacent residues under acidic conditions, and these intermediates may also tautermerize. Due to these difficulties, Siedler et al. [3] suggested the use of 4-vinylpyridine for the derivatization of the sulfhydryl group prior to acid hydrolysis. This method not only protects the cysteine from other reactions, but also prevents thiazoline formation and suppresses the strong sequence dependent rate of cysteine racemization during hydrolysis.

There are two techniques for resolving chiral compounds: direct and indirect methods. In direct methods, racemic *S*-pyridylethylated cysteines have been analyzed following acid hydrolysis by chiral gas chromatography or capillary zone electrophoresis [3]. In these analytical systems, specialized columns are required. These columns are usually not readily available. Furthermore, in gas chromatographic systems, the samples must be made volatile, a complex and sometimes difficult procedure.

In indirect methods, the chiral amino acids are first derivatized to form diastereomers. As a result of the formation of diastereomers, separation can be achieve with conventional high-performance liquid chromatography (HPLC).

Since Marfey's reagent, FDAA (1-fluoro-2-4-dinitrophenyl-5-L-alanine-amide) [4] has proven to be a

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0021-9673/98/$19.00
PII: S0021-9673(98)00289-1

successful derivatizing reagent for the chromatographic analysis of the complex mixtures of amino acid enantiomers, it was chosen to derivatize the *S*-pyridylethylated cysteines of our peptide hydrolysates. Although good resolutions for the separation of racemic mixtures of most Marfey's derivatized amino acids have been shown, studies involving FDAA-derivatized *S*-pyridylethylated cysteine have not been reported.

This report describes: (A) the disulfide reduction and vinylpyridylation for the stabilization of cysteine, (B) the derivatization of the peptide hydrolysate with Marfey's reagent for subsequent liquid chromatographic analysis, and (C) the results obtained for the somatostatin analogs.

2. Experimental

2.1. Materials

4-Vinylpyridine, (Aldrich), tris(2-carboxyethyl)-phosphine, (Aldrich), 6 *M* HCl (Pierce), Tris (Bio-Rad), citric acid (Mallinckrodt), Marfey's reagent (FDAA) (Sigma), sodium hydrogencarbonate and sodium hydroxide were used as received.

Ten synthesized somatostatin analogs (Sadat [5]) were used as received.

The general formula for these analogs is: A*x*-*c*[D/L-Cys-A*y*-D/L-Cys]-A*z*-NH_2 where: A=amino acid, x=1–2 amino acid residues, y=2–4 amino acid residues, z=1–2 amino acid residues.

2.2. Instrumentation

System A (a semi-preparative HPLC system) consists of two Rainin HPXL pumps, a (Model 7129) Rheodyne injector, a Rainin detector (Dynamax UV-C), and a Rainin Dynamax Model FC-1 fraction collector. The signals were recorded via a strip chart recorder (Houston Omni scribe).

The method is as follows: column: Zorbax C_8 25×1 cm; mobile phase: buffer A: 0.1% trifluoracetic acid (TFA) in water, buffer B: 0.1% TFA in 80% aqueous acetonitrile (ACN)–water (80:20), gradient 5% B to 60% B in 30 min; flow-rate: 5 ml/min; detection: 220 nm.

System B (analytical system) consists of two Rainin HPXL pumps (computer controlled), a Rainin Dynamax autosampler A1-1A, an Applied Biosystems 785A UV detector and a Macintosh Quadra 630 computer for data collection and analysis (Rainin dynamax software).

The method is as follows: column: Nucleosil C_{18} 5 μm, 100 Å, 250×4.6 mm; mobile phase: buffer A: 0.1% TFA in water, buffer B: 0.1% TFA in ACN, gradient 11% to 66% B in 50 min; flow-rate: 1 ml/min; detection: 340 nm.

2.3. Procedure

The procedure to determine the chirality of cysteine requires five steps: (A) reduction and derivatization of cysteines in the somatostatin analogs, (B) purification of *S*-β-(4-pyridylethylated) cysteine somatostatin analogs, (C) hydrolysis of the *S*-β-(4-pyridylethylated) cysteine somatostatin analogs, (D) derivatization of the hydrolyzed *S*-β-(4-pyridylethylated) cysteines, and (E) HPLC analysis of the diastereomers of pyridylethylated cysteines.

(A) Approximately 1 mg of somatostatin analog in 1 ml degassed and argon-saturated 10 m*M* Tris–citrate buffer, pH 5.0 was reduced with an equivalent (10% excess) of tris(2-carboxyethylphosphine) at 37°C for 45 min. One equivalent of 4-vinyl pyridine/SH was added and allowed to react in the dark for 2 h. The product is *S*-β-(4-pyridylethylated) cysteine peptide.

(B) *S*-β-4-Pyridylethylated cysteines were purified using a Zorbax C_8 25×1 cm column. The method is described in Section 2.2. The *S*-β-(4-pyridylethylated) peptide collected was lyophilized.

(C) The lyophilized powder was hydrolyzed with 1 ml 6 *M* HCl in a sealed evacuated ampoule at 110°C for 22 h. The hydrolyzed samples were then evaporated to dryness with a gentle stream of nitrogen and reconstituted with 0.2 *M* sodium hydrogencarbonate solution to give an amino acid concentration of 10 m*M*. The pH was adjusted to 7.5–8.5 with 1 *M* NaOH.

(D) A 1.1 equivalent amount (with respect to the amino acids in the hydrolyzate) of Marfey's reagent was added. The reaction was allowed to proceed at 40°C for 1 h. The solution was quenched with an equivalent volume of 0.2 *M* HCl.

(E) About 30 μl of the quenched samples were analyzed directly by reversed-phase HPLC (method is described in Section 2.2). The DL isomer separated

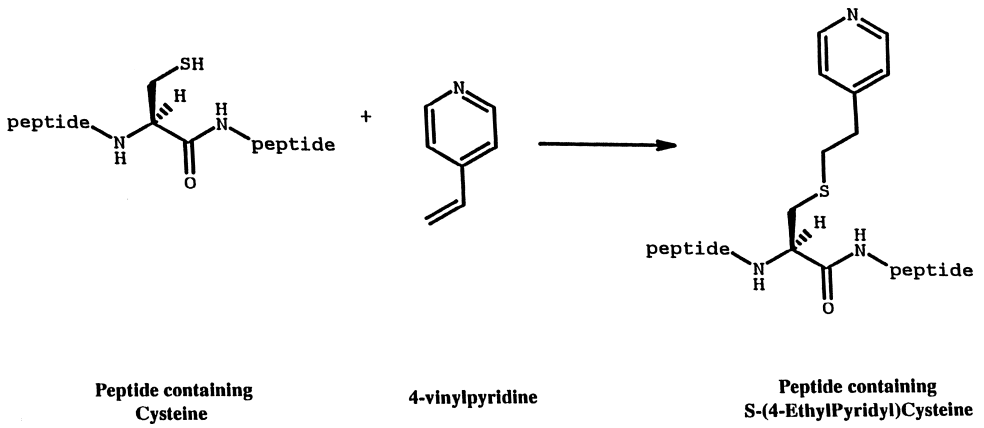

Fig. 1. Vinylpyridylation of cysteine.

before the LL isomer. Quantitation was done by comparing the standards made using *S*-β-(4-pyridylethylated cysteines) obtained from Aldrich.

3. Results and discussion

Fig. 1 shows the reaction of cysteine residues with 4-vinylpyridine. Although the reaction as described by Siedler et al. is fast, excess 4-vinylpyridine may form side products. These may complicate the hydrolysis and the chromatography. To reduce these complications, purification of the active component [(*S*-4-ethylpyridyl)cysteine peptide] was performed.

Purification, as indicated in Section 2.2, was done

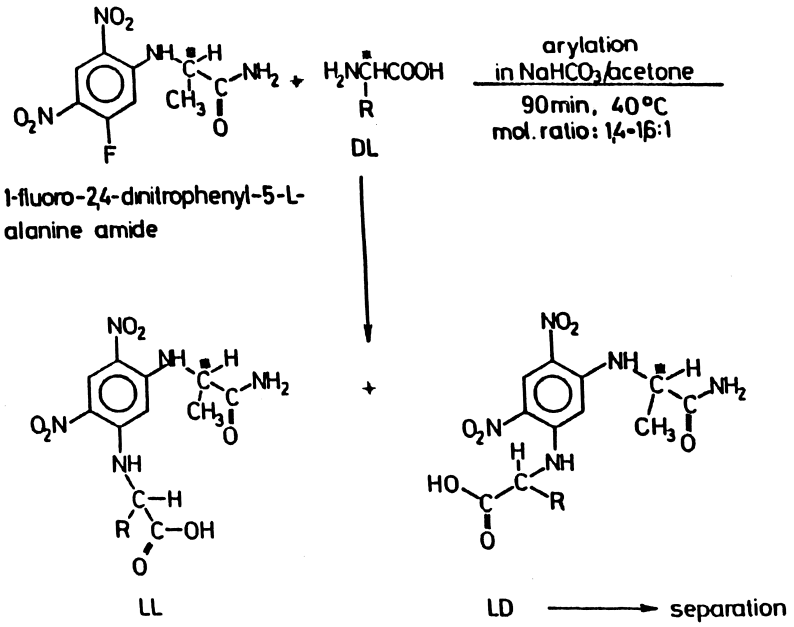

Fig. 2. Reaction of DL-amino acids with Marfey's reagent.

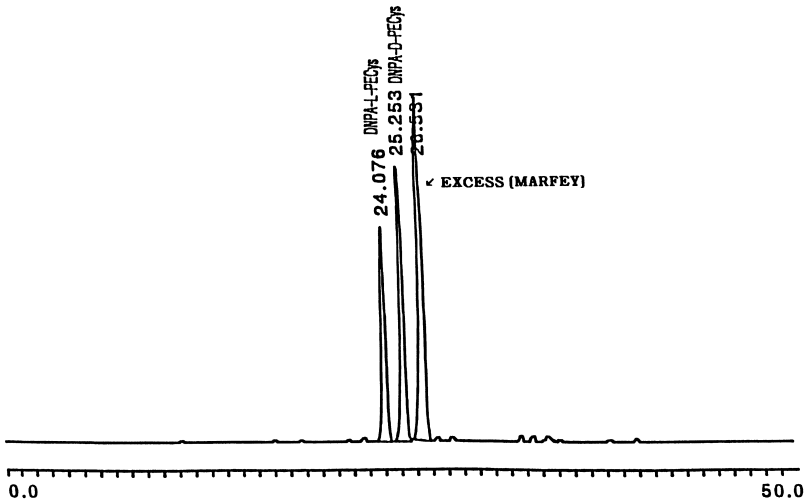

Fig. 3. Derivatization of standard VP-D/L-Cys (50:50 mole ratio solution of D/L-Cys-pH 8) with Marfey's reagent; 11–66% B in 50 min Nucleosil C_{18}, 5 μm, 100 Å; 30 μl injected; sampling interval 0.1 s. Time scale in minutes.

on a C_8 semi-preparative column. The *S*-(4-ethylpyridyl)cysteine peptide elutes much later than impurities.

Fig. 2 illustrates the derivatization reaction of an amino acid with Marfey's reagent. The reaction with *S*-(4-pyridylethylated)cysteine also proceeds in a similar manner as other non-derivatized amino acids. This is shown in a chromatogram in Fig. 3 obtained for the reactions with standards [*S*-(4-ethylpyridyl)cysteine isomers obtained from Sigma]. No new peaks were found.

The analytical HPLC method used in the determination of the chirality of cysteines is as described in Section 2.2. The analysis was performed at 340 nm to avoid detection of unreacted starting materials. Figs. 4 and 5 show the chromatograms

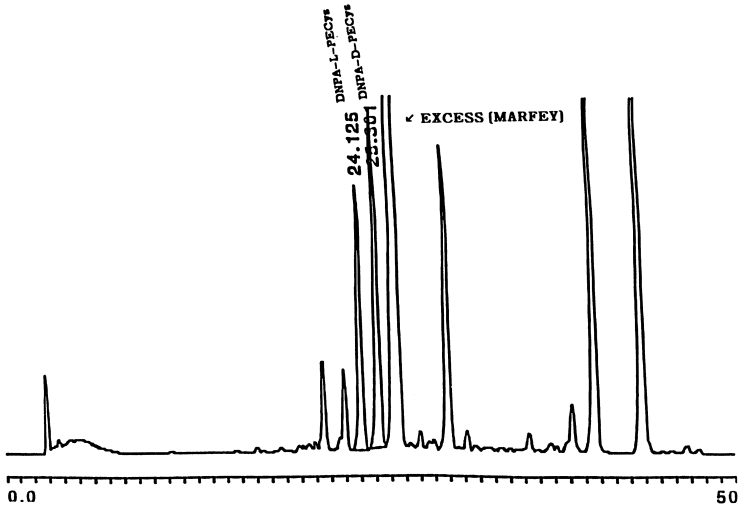

Fig. 4. Analog 4. After reduction, vinylpyridylation of Cys residues, hydrolysis and reaction with Marfey's reagent; 11–66% B in 50 min Nucleosil C_{18}, 5 μm, 100 Å; 30 μl injected; sampling interval 0.1 s. Time scale in minutes.

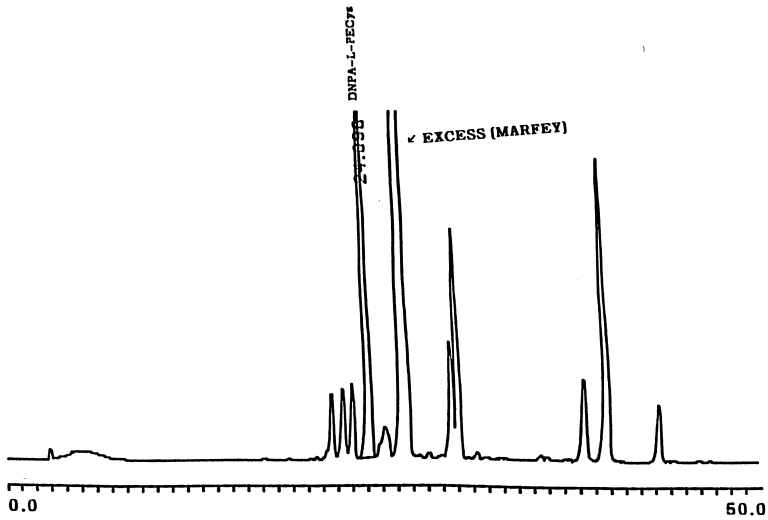

Fig. 5. Analog 5. After reduction, vinylpyridylation of Cys residues, hydrolysis and reaction with Marfey's reagent; 11–66% B in 50 m Nucleosil C_{18}, 5 μm, 100 Å; 30 μl injected; sampling interval 0.1 s. Time scale in min.

obtained for samples analog 4 and analog 5, respectively. As shown in Fig. 4 the diastereomers are well resolved for easy quantitation.

Ten analogs of somatostatin, each containing two cysteines, were analyzed. The results are shown in Table 1. There is an agreement between the theoretical and actual results (the results calculated are with in ±15%). These results also show that the derivatization of cysteine with 4-vinylpyridine protects the chirality of cysteines during hydrolysis. No measurable ''hydrolysis–racemation'' of the cysteines were observed for peptides which contain only one enantiomer. Even though, as shown in Fig. 5 (a peptide with only L-cysteines) two peaks were observed, the D isomer peak is very small. This D isomer peak is likely due to the impurity (D isomer) in the reagents used during synthesis rather than a ''hydrolysis racemate''. This is also evident in other peptides synthesized with one cysteine enantiomer. This method is safe and adaptable to protect and to retain the enantimeric composition of the cysteines.

Table 1
Comparison of ratios of L- and D-cysteine isomers obtained experimentally to those of theoretical values in these somatoststin analogs

Peptide	Ratios of L:D Cys determined	Expected
Analog 1	2:0	2:0
Analog 2	1:1	1:1
Analog 3	1:1	1:1
Analog 4	1:1	1:1
Analog 5	2:0	2:0
Analog 6	1:1	1:1
Analog 7	2:0	2:0
Analog 8	1:1	1:1
Analog 9	1:1	1:1
Analog 10	0:2	0:2

4. Conclusions

The results as shown indicate that 4-vinylpyridine protected and retained the chirality of cysteines during the hydrolysis reaction. The pyridylethylated cysteines are also easily derivatized with Marfey's reagent to yield the (DL and LL) diastereomers for subsequent HPLC analysis.

The HPLC separation procedure for resolving the DL and LL diastereomers uses conventional achiral columns. Baseline resolutions were obtained for these diastereomers. The peaks are easily identifiable and the results are quantitative and sensitive.

Although there are two derivatization steps in the determination, the reactions involved are straightforward and fast. The procedure as described may be

adapted for the determination of the chirality of cysteines for any cysteine containing peptide.

References

[1] G. Szókán, S. Hadfi, K. Krizsán, A. Liembeck, I. Krecz, M. Almás, C. Somlai, J. Liq. Chromatogr. 17 (1994) 2759–2775.
[2] W. Woimode, H. Frank, G.J. Nicholson, E. Bayer, Chem. Ber. 111 (19xx) 3711-3718.
[3] F. Siedler, J. Peptide Sci. 2 (1996) 271–275.
[4] P. Marfey, Carlsberg Res. Commun. 49 (1994) 591–596.
[5] D. Sadat, Biomeasure, Milford, MA, personal communication.