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Configuration and Racemization Determination of Cysteine Residues in Peptides by Chiral Derivatization and HPLC: Application to Oxytocin Peptides

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Abstract: An improved RP-HPLC method was developed for the determination of the configuration and stereochemical purity of cysteine residues in peptides. The method consists of oxidation of cysteine and cystine residues to cysteic acid, followed by hydrolysis and pre-column chiral derivatization with Val-Marfey's reagent. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

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INTRODUCTION

Chiral liquid chromatography has been widely used to check the enantiomeric purity of synthetic, biologically active peptides. This method also allows the determination of the absolute configuration of amino acids in unknown, isolated peptides, glycopeptides and peptide antibiotics. It is well known that, regardless of the thiol-protecting groups used in the synthesis of cysteine peptides, the easy racemization of this residue is a serious problem during both liquid and solid phase peptide synthesis. Because of the difficult analytical quantification of the enantiomeric ratio of cysteine, proper attention has not been paid to this problem in the past. The chiral analysis of hydrolysed cysteine peptides is complicated by the sequence-dependent, high rate racemization of cysteine during acidic hydrolysis. Unfortunately, an exact chiral Edman sequence analysis has not yet been elaborated.

However, sophisticated chiral gas chromatographic and capillary electrophoretic methods have been described for the exact determination of cysteine racemization [1].

Because of the great popularity and routine usage of Marfey's derivatization in the analysis of the optical purity of the constituting amino acid residues in peptides, we tried to apply this method also for cysteine containing peptides. This effort is supported by the efficient RP-HPLC separation of all amino acid Marfey derivatives (for reviews see [2–5]). The L- and D-amino acid Marfey derivatives can be identified by co-injection of standard derivatized D- and L-amino acids. The chromatographic reciprocity principle makes it possible, using both enantioisomeric reagents, to determine the opposite stereoisomer's retention time without measuring the corresponding authentic sample.

Another advantage of Marfey's reagent is the possibility to increase its hydrophobicity (thus increasing α and R_S of the derivatives), by replacing the chiral selector Ala-NH_2 of the reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) with other amino acids, such as Leu-NH_2 or Val-NH_2 (for reviews see [6–9]).

This method has been introduced and improved using LC/MS and the new technique called

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'advanced Marfey's method' (for reviews see [9–13]) was successfully applied to the analysis of an anabaenopeptide produced by a cyanobacterium [9], and to the determination of the absolute configuration of 3-amino-6-hydroxy-2-piperidone, one of the constituent amino acids in aerugino peptin [11]. Marfey's method was also used to determine the enantiomeric purity of amino acid residues in subtractive Edman degradation of peptides [14].

The best approach of controlling the enantiomeric purity of cysteine and cystine peptides is to apply epimeric cysteine-containing peptides as references. This route has been used rarely for racemization studies of Cys peptides because of the lack of reference peptides and the low efficiency of epimeric peptide separation (for reviews see [15–19]).

Comparative racemization studies, using the Marfey's method and the approach of separating epimeric mixtures of oxytocin and other peptides containing cysteine or cystine, were conducted.

Table 1 Comparison of Chromatographic Data for Derivatized Racemic Cysteic Acid

Chiral derivatization reagent	t_R		k'		α	R_S	HPLC columns	Eluents	Detection UV (nm)
	L	D	L	D					
Ala-Marfey's reagent (FDAA)	1.6	1.6	0.33	0.33	1.00	0.00	(q)	(t)	340
Val-Marfey's reagent (FDVA)	8.8	12.0	7.8	11.0	2.15	3.4	(p), (q)	(t)	340
OPA/*N*-IBLC	22.0	24.4	10.0	11.2	1.12	1.59	(n)	(r)	220
GITC	85.8	91.8	34.7	37.2	1.10	1.66	(m)	(s)	250
BGIT	56.6	59.2	19.2	20.1	1.05	1.53	(m)	(s)	231
Fmoc-Val-F	58.3	61.7	44.6	47.9	1.04	1.23	(n)	Gradient	254

(m) Beckman ODS column (125 × 4.6 mm. i.d.); (n) Hypersil, ODS, column-5 μm (125 mm × 4.6 mm); (p) column C18 Lichrospher-100 (100 × 4 mm); (q) column C18 Hypersil-6 (125 × 4.6 mm); (r) methanol/(1/15 M) Na_2HPO_4 (pH 7.3); (s) methanol/0.02 M KH_2PO_4 (pH 2.8); (t) methanol/acetonitrile/0.02 M ammonium acetate buffer (pH 4).

Table 2 Description of Methods for Optical Purity and Absolute Configuration Determination in the Case of Cysteine and Cystine Containing Peptides

Compound type and method	General structure of studied compounds	Processes order	Final products	Examples of studied compounds
(a) Cysteine containing peptides	X-Cys-Y	1. Oxidation 2. Hydrolysis 3. Val-Marfey derivatization 4. HPLC	FDVA-Cys FDVA-X FDVA-Y	(1) Boc-Glu-Cys-Gly-OH (2) Glu(Cys-Gly) glutathione (3) 1-D-6-L-oxytocin (4) 1-L-6-D-oxytocin (5) 1,6-L-oxytocin (6) 1,6-D-oxytocin
(a) Cystine containing peptides	X-Cystin-Y	1. Oxidation 2. Hydrolysis 3. Val-Marfey derivatization 4. HPLC	FDVA-Cys FDVA-X FDVA-Y	(1) MCD peptide (2) Somatostatin
(b) Peptides containing *N*-terminal cysteine	Cys-X-Y	1. Val-Marfey derivatization 2. Oxidation 3. Hydrolysis 4. HPLC	FDVA-Cys X Y	(1) 1-D-6-L-oxytocin (2) 1-L-6-D-oxytocin (3) 1,6-L-oxytocin (4) 1,6-D-oxytocin

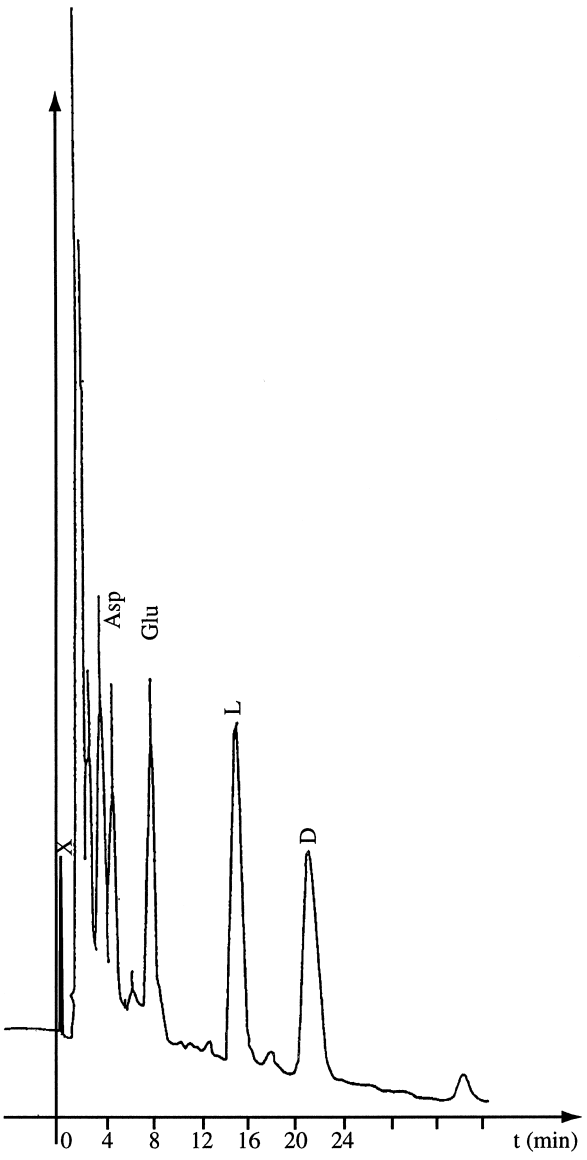

Figure 1 Chromatographic pattern of chiral amino acid analysis of oxytocin derivatized with Val-Marfey's reagent. Column: ODS-Hypersil-6 (125 × 4.0 mm); eluent: methanol/0.02 M sodium acetate buffer (pH 4) – 60:40 (v/v); flow rate: 1.0 mL min^{-1}; detection: UV-340 nm.

MATERIALS AND METHODS

Materials

Oxytocin (H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2); (1-D-Cys)-oxytocin; (6-D-Cys)-oxytocin; (1,6-bis-D-Cys)-oxytocin were synthesized in solution by a patented procedure of Chemical Works of Gedeon Richter Limited, Budapest, Hungary.

Boc-Glu-Cys-Gly-OH and Glu(Cys-Gly) were a gift from REANAL-Fine Chemicals Company, Budapest, Hungary.

Mass cell degranulating peptide (MCD peptide) and somatostatin were received from Chemical Works of Gedeon Richter Limited.

The solvents *n*-hexane, dichloromethane, methanol and acetonitrile were all of HPLC grade, and were purchased from Sigma Chemical Company

Reagents Used and Their Suppliers

1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA – Alanine Marfey's reagent) (Sigma); 1-fluoro-2,4-dinitrophenyl-5-L-valine amide (FDVA – Val-Marfey's reagent) (Fluka); 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) (Fluka); 2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl isothiocyanate (BGIT) (Fluka); 9-fluorenyl-methyl-oxy-carbonyl-valinefluoride (Fmoc-Val-F) was received from M. Bienert (Inst. Mol. Pharmacology, Berlin, Germany); ortho-phthalaldehyde (OPA) and *N*-isobutyryl-L-cysteine (*N*-IBLC) (Merck).

Methods

Oxidation. Cystine peptides were prepared by oxidation of cysteine peptides using 30% H_2O_2 in acetic acid, as described in the literature [20].

Hydrolysis

One to two mg of hydrolysed sample was dissolved in 0.5 mL trifluoroacetic acid/6N HCl 1:1 mixture and kept at 105°C for 48 h in sealed ampoules. After evaporation and drying in vacuum, the residues were dissolved in 100 µL of methanol/acetonitrile 2:1 mixture or derivatized and used for HPLC analysis [21].

Derivatization with Marfey's Reagents

Derivatization of amino acids (Method a) (4). Derivatization was carried out according to Marfey's standard procedure with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide or 1-fluoro-2,4-dinitrophenyl-5-L-valine amide. The peptide-hydrolysate prepared from 2–5 µmol sample was dissolved in 100 µL 0.5 M $NaHCO_3$ solution and 200 µL of Marfey's reagent solution (1% solution in acetone; w/v) was added. The solution was incubated at 40°C for 90 min, cooled to ambient temperature, and mixed with 25 µL 2 M HCl. After dilution with 20 times excess of methanol or

Table 3 Determination of Cysteine Configuration in Epimeric Oxytocines Based on Enantiomeric Composition of Derivatized Compounds

Compounds	Cysteic acid configuration			
Method	(b)		(a)	
	D	L	D	L
Oxytocin	−	+	−	+
(1-D-Cys)-oxytocin	+	−	+	+
(6-D-Cys)-oxytocin	−	+	+	+
(1,6-bis-D-Cys)-oxytocin	+	−	+	−
Unknown 'oxytocin-like' compound	+	−	+	+

+ Presence; − absence.

another appropriate eluent, 10–20 μL aliquots were injected directly to the column.

Derivatization of peptides (Method b) (3). A milligram, containing *N*-terminal cysteine (e.g. oxytocin) was kept with 10 μmol reagent in 50 μL 0.5N $NaHCO_3$ and 100 μL acetone for 4 h, according to the general Marfey's method. After drying in vacuum, the solid residue was dissolved in 1 mL acetic acid. Further, 0.5 mL 30% H_2O_2 were added as oxidation agent. It was held for 4 h at room temperature, and dried again in vacuum over KOH. The product was hydrolysed, as described above, and used for HPLC analysis.

Pre-column derivatization of cysteic acid with orthophthalaldehyde and N-isobutyryl-L-cysteine (OPA/N-IBLC) (8). One milligram of sample was hydrolysed for 24 h in a closed vial using 250 μL 6N HCl at 110°C. The solution was evaporated to dryness in vacuum, then the residue dissolved in 100 μL 0.1N HCl. From this solution, a 6-μL aliquot was mixed with 15 μL 0.4N sodium borate buffer (pH 10.4) and 3 μL OPA/N-IBLC reagent (20 mM *N*-IBLC and 170 mM OPA in 1 M potassium borate buffer – pH 10.4) for 3 min, then injected into the column.

Derivatization of L,D-cysteic acid with isothiocyanate reagents (GITC or BGIT) (22). Two milligrams of L,D-cysteic acid was dissolved in 2 mL of 50 % (v/v) aqueous acetonitrile containing 0.4% (w/v) triethylamine. A 25 μL aliquot of this stock solution was mixed with 50 μL of a 0.2% (w/v) solution of GITC or BGIT reagent in acetonitrile. The reaction mixture was stirred at room temperature for 1 h, and 10 μL was injected onto the column.

Derivatization of L,D-cysteic with Fmoc-Val-F (23). For this, 2.5 μmol of free amino acid were dissolved in 100 μL distilled water, and then mixed with 3.75 μmol of Fmoc-Val-F (as reagent) dissolved in 200 μL acetone or DMSO. The pH was adjusted to 8.0 by 40 μL 0.5N $NaHCO_3$, the reaction mixture was left overnight at room temperature, then the derivatization reaction was checked by TLC, with butanol:acetic acid:water/40:1:1 (v/v/v) mobile phase, and finally, 10–20 μL aliquot was injected onto the column.

High Performance Liquid Chromatography

Separations were performed on a Knauer HPLC system consisting of two Model 64 pumps, a Model 50B gradient programmer and a Model 87.00 spectral photometer. The HPLC systems were operated isocratically with flow rates between 0.8 and 2.0 mL min^{-1} or with gradient elution. The chromatographic data were collected and processed with CHROMAPEX software (Data Apex Limited, Czech Republic).

Table 4 Retention Data (*k'*-Retention Factor) of Derivatized Oxytocins

Compound	Ala-Marfey derivative	Val-Marfey derivative
Oxytocin	4.0	12.6
Isolated 'impurity'	5.0	16.0
1-D-Cys-oxytocin	5.0	16.0

Column: μ Bondapak C18. Eluent: acetonitrile/ammonium acetate buffer. Detection: UV at 340 nm.

RESULTS AND DISCUSSION

It was found that racemization of cystine in oxytocin and related peptides during Marfey's chiral amino acid analysis gives rise to further problems. Racemization of cystine is difficult to evaluate, because diastereomers might also be formed. After reduction (e.g. with dithiothreithol) other problems appeared because of incomplete derivatization of cysteine (*N*-arylation, *S*-arylation, formation of bis-derivative and re-oxidation to cystine), which led to irreproducible results.

Therefore, various approaches to the modification of Cys before hydrolysis were considered: the oxidation to cysteic acid or the reduction to cysteine followed by alkylation with an alkylating reagent. The concept of disulphide interchange during conventional hydrolysis offers another approach to these analyses [1].

A method for the separation of *S*-protected cysteine Marfey's derivatives has been elaborated by our laboratory [5]. For example, the reduced cysteine units and original cysteine residues were transformed into 2-aminoethyl cysteine. However, the reagent is very toxic and during the reaction with Marfey's reagent, three derivatives can be formed via arylation of the α-amino and/or *S*-aminoethyl groups.

The transformation of cysteine residues into the stable cysteic acid helped to solve the problem.

In normal phase systems, the chromatography of suphonic acids is very difficult, usually because of tailing. By ion-pair chromatography, it is possible to improve hydrophobicity and inherently the peak shape, but the resolution is still low.

To eliminate the chromatographic disadvantage of cysteic acid, which is always an early eluting peak in RP-HPLC and to improve the enantiomeric separation, the following hydrophobic chiral derivatizing agents were used:

1. ortho-phthalaldehyde (OPA) with *N*-isobutyryl-L-cysteine (*N*-IBLC) additive [8];
2. 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) [22];
3. 2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl isothiocyanate (BGIT) [22];
4. Ala-Marfey's reagent, with alanine chiral selector [6,7];
5. Val-Marfey's reagent, with D- or L-valine chiral selector [6,7];
6. 9-fluorenylmethyl-oxy-carbonyl-valine fluoride (Fmoc-Val-F).

First we applied Brueckner's method using OPA and *N*-isobutyryl cysteine as chiral -SH additive.

The chiral isothiocyanate reagents with four *O*-acyl groups (acetyl or benzoyl) also increase the hydrophobicity of cysteic acid. It was found that both GITC and BGIT reagents are suitable for the separation of enantiomers, but the GITC reagent gives better separation than BGIT.

As expected, any *N*-protected and activated amino acid derivative, with a bulky hydrophobic group, can serve as chiral derivatizing agent. Many *C*-activated enantiomeric amino acid derivatives (active esters, anhydrides and halides) can be taken into account [23,24].

Fmoc-amino acid chlorides were introduced as a new class of highly-reactive acylating reagents in peptide synthesis [25], but in agreement with other data [26], we found that Fmoc-Val-F is a better chiral derivatizing agent for free amino acids such as cysteic acid than Fmoc-amino acid chlorides, or Fmoc-Val-OH together with DCCI. The Fmoc group increases the hydrophobicity and it is a good chromophor improving detection.

Results of HPLC studies are summarized in Table 1.

On the basis of the separation factors (α) and resolution (R_S), we selected advanced Marfey's reagent, containing Val-NH_2 as chiral selector instead of Ala-NH_2, for the separation of L- and D-cysteic acid (see Table 1). This replacement, according to Rekker hydrophobic fragment constants [27], means a $\Delta\varepsilon_f = 0.93$ hydrophobicity increase. The assay is reliable to 0.05% racemization. It was found that Ala-Marfey's reagent is suitable only for separation of homocysteic acid (which has one more CH_2 group).

Based on our results, two procedures are recommended (a and b) for configuration determination of peptides containing cystine and cysteine (see Table 2).

With method (a) the enantiomeric purity of all constituting amino acid residues can be determined. Figure 1 shows the resulting chromatographic pattern when applying method (a) to an oxytocin epimer. Generally, the Marfey derivative of the proteinogenic L-amino acids are eluted from the column before their corresponding D-isomer (Figure 1).

According to our experiments, Marfey's derivatization of peptides (e.g. oxytocin) with free α-amino group (method b) requires longer reaction time than that of amino acids. Instead of 90 min, the reaction time for peptides is 12–24 h and five times excess of

reagent is necessary. The retention time of peptides increases significantly, depending on the size of the peptide fragment. For the peptides studied (enumerated in Table 3), the retention time was two to three times longer than for free cysteic acid. Using Val-Marfey's reagent, the retention times vary between 4 and 20 min.

Table 3 summarizes the results on epimeric oxytocins. On the basis of the chromatographic patterns, using method (b) oxytocin and (6-D-hemi-Cys)-oxytocin samples gave only L-cysteic acid derivative, while (1-D-hemi)-oxytocin and (1,6-bis-D-Cys)-oxytocin produced D-cysteic acid derivative (Table 3).

The advantage of method (b) is the special targeting, because only the amino acid in position 1 gives Marfey-derivative, which is detected at 340 nm. In this way, the chromatographic pattern is much simpler. Using both methods (a) and (b), in the case of oxytocin-like peptides, we could differentiate cysteine residues at positions 1 and 6 and determine their configuration. We could quantitatively determine the racemization degree in epimerized oxytocin batches with an accuracy of 0.2–0.3%. The limitation of the method is reached when there are more than one Cys in the sequence of the peptide, except for the *N*-terminal, but with an adequate sequencing, this problem could also be solved.

Methods (a) and (b) were also applied for an isolated side product from an industrial scale batch of synthetic oxytocin. This component had the same molecular mass and amino acid composition as pure oxytocin. From the HPLC data (see Tables 3 and 4), it was concluded that the isolated side product is 1-D-Cys-oxytocin.

According to Marfey's chiral amino acid analysis during the synthesis of oxytocin racemization occurs only at the cysteine residues (Table 4).

CONCLUSIONS

Two step transformation (oxidation and hydrolysis) of cysteine and cystine residues in peptides and post-hydrolytic pre-column derivatization with advanced Marfey's reagent gives a very good separation of derivatized L- and D-cysteic acid residues. This method can be applied for determination of configuration and epimerization of cysteinyl residues in natural and synthetic peptides with a 0.2–0.5% detection limit.

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