Amino Acids (2008) 35: 445–450
DOI 10.1007/s00726-007-0577-1
Printed in The Netherlands

Amino Acids

Gas chromatographic analysis of amino acid enantiomers in Carbetocin peptide hydrolysates after fast derivatization with pentafluoropropyl chloroformate

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Received April 25, 2007
Accepted June 26, 2007
Published online August 28, 2007;

Summary. A novel sample preparation protocol for gas chromatographic (GC) analysis of amino acid enantiomers in peptides was developed. It comprises traditional acid hydrolysis, a novel treatment of the analytes with a fluoroalkyl chloroformate and GC/FID separation of enantiomers on a chiral capillary column. The major improvements consist in that the derivatization step proceeds in organic-aqueous media within seconds and the amino acid derivatives are volatile enough to suit the temperature range of the chiral Chirasil-Val® capillary column. The approach was found beneficial for chiral analysis of pharmaceutically important Carbetocin peptide.

Keywords: D,L-amino acids – Peptides – Chloroformates – GC – Chiral separation

Introduction

The examination of enantiomeric/diastereoisomeric purity of drugs is currently accepted as one of the most important steps when studying pharmacokinetic, pharmacodynamic and xenobiotic properties of chiral drugs (Clark and Mama, 1989; Maier et al., 2001). Stereoisomers of drugs can produce different therapeutic (or adverse) effect (Ariëns, 1986; Maier et al., 2001; Gübitz and Schmid, 2006). The initial step involves the development of a suitable method for the separation of enantiomers or diastereoisomers. A number of peptides have been applied in medical care. Determination of their chiral purity is, therefore, an important prerequisite required by regulatory authorities.

Numerous analytical methods have been developed for peptide chiral purity determination. The chiral analyses of peptides consist of two basis steps: 1) hydrolysis of peptide to free amino acids, 2) analysis of released amino acids themselves. The time of hydrolysis required for complete liberation of the amino acids from peptides depends on the nature of their linkages. For this reason, 3 or 4 hydrolyses are often performed with different hydrolysis times in order to determine the optimum values for present AAs. Chiral analyses of amino acids are mostly carried on by chromatographic or electrophoretic methods. Reversed-phase high performance liquid chromatography methods can resolve D- and L-amino acids after their derivatization with 1-fluoro-2,4,-dinitrophenyl 5-L-alanine amide (FDAA, Marfey's reagent) (Goodlett et al., 1995; Hess et al., 2004; Sohda et al., 2005), with 2,3,4,6-tetra-O-acetyl-beta-D-glucopyranosyl isothiocyanate (GITC), (S)-N-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester (S-NIFE), with o-phthalaldehyde-isobutyryl-L-cysteine (OPA-ILBC) (Brückner et al., 1995; Reichelt et al., 1999; Sohda et al., 2005), with 2-chloro-4-methoxy-6-(4-methoxy-1-naphthyl)-[1,3,5]triazine (CMMNT) (Brückner and Wachsmann, 2003) or with fluorescent chiral Edman-type reagents, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole [R(−)- and S(+)-NBD-PyNCS] and 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole [R(−)- and S(+)-DBD-PyNCS] (Toyooka and Liu, 1995). The chiral resolution of amino acids released from peptides was achieved also after derivatization with chiral reagent (+)- or (−)-1-(9-anthryl)-2-propyl chloroformate (APOC) using micellar electrokinetic chromatography (MEKC) with laser-induced fluorescence detection (Thorsén et al., 2001). The traditional derivatization

methods for gas chromatographic AA enantiomers determination in peptide hydrolysates are based on two-step procedures. These involve esterification with propanol (PrOH/HCl) (Bayer et al., 1987) or isopropanol (iPrOH/HCl) (Fu et al., 1998; Kawulka et al., 2004), and acylation with anhydrides, e.g., of pentafluoropropionic (Bayer et al., 1987; Kawulka et al., 2004) or trifluoroacetic acid (Fu et al., 1998; Nokihara and Gerhardt, 2001). The derivatized AA enantiomers are then separated on Chirasil-Val® column. A qualitative thin-layer chromatographic (TLC) method was developed for chiral analysis of α-amino acids in small peptides. Following hydrolysis, the compounds were treated with dansyl chloride (DNS-Cl), the derivatives were purified by TLC, and the analysis was carried out on a reversed-phase TLC using mobile phases with chiral β-cyclodextrine (β-CD) (LeFevre et al., 2000). Analyses of the dansylated peptide hydrolysates were done by chiral capillary electrophoresis (CE) (β-cyclodextrine was used as chiral selector) (Moore et al., 1999). CE was also applied for separation of diastereomers of amino acids in hydrolyzed peptides prepared by derivatization with a fluorescent chiral reagent R-(−)- or S-(+)-NBD-PyNCS (Liu et al., 1998). Tandem mass spectrometry (MS/MS) was also applied for qualitative and quantitative chiral analyses of individual amino acids residues in polypeptides, in combination with nanoflow reversed-phase liquid chromatography (Adams and Zubarev, 2005).

Most of above-mentioned methods are tedious, time-consuming, and requiring anhydrous conditions and heating. We propose in this paper a fast and simple method of D/L-amino acids determination. The reaction proceeds in aqueous medium at room temperature with a novel derivatization reagent, 2,2,3,3,3-pentafluoropropyl chloroformate PFPC (Hušek et al., 2007). The AA enantiomers are separated on a Chirasil-Val® column. The approach was developed and applied to the determination of molar ratio of L- and D-enantiomers of amino acids in the peptide D-Asn-Carbetocin, used as a stimulating lactation drug.

Materials and methods

Materials

D- and L-amino acids (purity >99.5%), pyridine, 2,2,4-trimethylpentane (isooctane) and thioglycolic acid, all in best available grade, were delivered from Sigma-Aldrich (Prague, Czech Republic). Sodium hydroxide was obtained from Lachema (Brno, Czech Republic, p.a. grade); dichloromethane was from Merck (Darmstadt, Germany). The 2,2,3,3,3-pentafluoro-1-propanol (PFP) was obtained from Fluorochem Ltd. (Glossop, UK), PFPC was synthesized in situ (Eckert, 1987) in the Biology Centre (České Budějovice, Czech Republic).

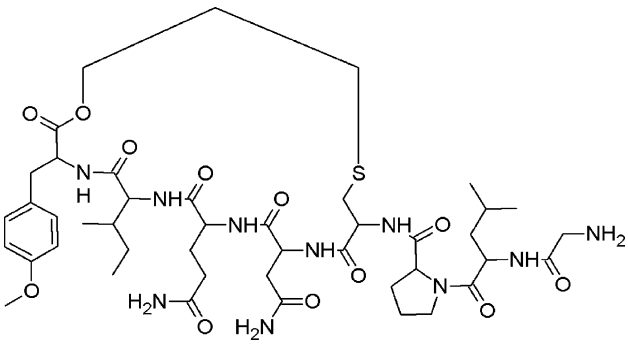
Standard solutions of D- and L-amino acids were prepared in 0.1 M HCl. Isoleucine (Ile), leucine (Leu), cysteine (Cys), proline (Pro), and O-methyl-tyrosine (MeOTyr) in the concentrations of 20 nmol/l and 100 nmol/l, aspartic acid (Asp) and glutamic acid (Glu) in 20 nmol/l and 50 nmol/l. Mixtures of D:L amino acids in ratios (nmol) 1:99, 5:95, 10:90, 20:80, 25:75 (only for Asp) were also prepared. Standards of Asp and Glu were used instead of Asn, Gln, because after hydrolysis only Asp and Glu is possible to detect.

Carbetocin peptide (L-Asn-Carbetocin) (purity >97%) and its D-Asn-Carbetocin impurity (purity >96%) were provided by Dr. Martin Flegel, Polypeptide Praha Ltd (Barth et al., 1981; Cort et al., 1982). According to the producer declaration Carbetocin peptide contains less than 1% of each corresponding amino acid D-enantiomer. L-Asn – and D-Asn Carbetocins were dissolved in 6 N HCl (final concentration 1.77 nmol/μl) containing 1% of thioglycolic acid added in order to prevent oxidation of Cys during hydrolysis.

Carbetocin is the octapeptide with the following composition:

Butyryl-MeOTyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 (Sulfide bond between Butyryl-4-yl and Cys), IUPAC Name: (2S)-1-[(3S,6S,9S,12S,15S)-12-[(2S)-butan-2-yl]-9-(2-carbamoylethyl)-6-(carbamoylmethyl)-15-[(4-hydroxyphenyl)methyl]-16-methyl-5,8,11,14,17-pentaoxo-1-thia-4,7,10,13,16-pentazacycloicosane-3-carbonyl]-N-[(1S)-1-(carbamoylmethylcarbamoyl)-3-methyl-butyl]pyrrolidine-2-carboxamide.

The structure of Carbetocin is the following (Scheme 1):



Scheme 1. Structure of Carbetocin peptide

GC analysis

Shimadzu GC 17A apparatus with AOC-20i Auto injector and flame ionization detector, all controlled by a Class VP software from Shimadzu (Japan), were employed for the analyses. The separation of AA enantiomers was carried out on the Chirasil-Val capillary column (25 m × 0.25 mm, 0.16 μm film thickness; Alltech, Deerfield, IL, USA). Helium carrier gas flow rate was 2 ml/min. The injector and detector temperatures were 240 and 250 °C, respectively. The programmed temperature operation started at 80 °C, followed by a rise of 10 °C/min to 110 °C, which of 0.5 °C/min to 123 °C, and a final one of 2.5 °C/min to 205 °C, held for 2 min. After 2 min of equilibration time, 1 μl sample was injected in splitless mode, splitless time was 0.2 min.

Procedure

Hydrolysis was carried out in glass vials 80 × 7 mm. In order to monitor the racemization, 100 nmol of each tested amino acid, L- and D-forms separately, and 100 μl of peptide solutions, L-Asn- and D-Asn-Carbetocins separately, were placed into vials. Vials were filled with nitrogen, sealed using glass-blowing burner and kept at 110 °C for 12, 18 and 24 h, each experiment in triplicate including blank determination.

Following hydrolysis, the sample was evaporated to dryness under a stream of nitrogen. The dry residue of released amino acids (or amino acid standards) was covered with 100 μl of aqueous 50 mmol/l sodium hydroxide-pyridine, 20:1 (v/v). The aqueous phase was exposed to extractive

alkylation upon admixing 25 µl of a mixture of isooctane, PFP and PFPCF (7:1:4). The content was vortex-mixed for about 5 sec till clearing the organic phase. After addition of 100 µl of isooctane and a brief vortexing, a 1-µl aliquot of organic phase was subjected to GC/FID analysis.

Calibration curves

Calibration curves reflecting dependence of the peak heights of D-enantiomer $*$ 100/(D + L) to amount of D-enantiomer [%] were established for all measured amino acids using mixtures of D- and L-AAs (1:99, 5:95, 10:90, 20:80, 25:75 (only for Asp)).

Results and discussion

Derivatization

Simultaneous derivatization of amino and carboxylic groups with chloroformates proceeds readily in aqueous media at room temperature, in presence of pyridine and the organic phase containing the reagent. Previously used derivatization methods for GC AA enantiomer separations required time-consuming two step procedures at elevated temperatures and a dry residue.

AA derivatives of PFPC were found to be sufficiently volatile to elute within the temperature range of the Chirasil-L-Val stationary phase. The PFPC derivatives of AAs were used for chiral determination of D/L-amino acids in peptides for a first time.

Calibration curves

The calibration plots denote linear relationships between *x* (amount of D-enantiomer in %) and *y* (the peak heights of D-enantiomer $*$ 100/(D + L)). Correlation coefficients (r^2) ranged from 0.9950 for Cys to 0.9989 for Leu.

Refinement of hydrolysis conditions, reproducibility

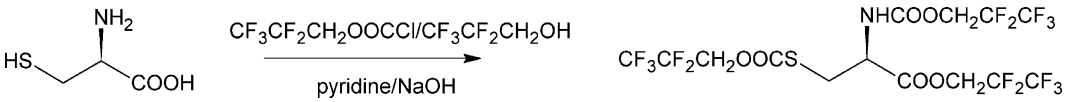
The time of hydrolysis required for complete liberation of the AAs from peptides depends on the nature of their linkages. Peptide bonds involving isoleucine or valine require longer hydrolysis time to be cleaved (Gehrke et al., 1987).

The relative content of the minor AA forms were counted from calibration plots. We tested hydrolysis at three different periods (12, 18 and 24 h) and found that hydrolysis times of 12 and 18 h are insufficient for complete hydrolysis (controlled by LC/MS, data not presented). As a result, 24-hour hydrolysis was employed. However, a partial decomposition of MeOTyr to Tyr (cleavage methyl group) was observed under such conditions of hydrolysis.

To determine reproducibility, hydrolysis of both peptides was processed six times and relative standard deviations (RSD) of D/L-AAs ratios were calculated (Table 1). The values of RSD ranged from 1.11 (Glu in L-Asn) to 3.74% (Cys in D-Asn).

Racemization

The racemization of AAs must always be expected during chemical treatments, such as acid hydrolysis, or even during storage. D-AAs may arise from inversion of configuration of peptide bound amino acids during the initial period of hydrolysis and later on from racemization of free AAs. The rates of inversion of *N*-acyl or peptide



Scheme 2. Conversion of amino acid to its derivative after treating with PFPC

Table 1. The results of the D:L-ratios of AAs determinations

Amino acid	Resolution	Temperature [°C]	RSD [%] of ratio D-/L-AA ($n = 6$)		Racemization [% of D-AAs]		
			L-Asn-Carb.	D-Asn-Carb.	Racemization gross (D-Asn-Carbetocine)	Due to hydrolysis	Net
Isoleucine	3.23	110					
Leucine	3.78	110	2.25	1.92	3.03	2.8	0.23
Aspartic acid	1.52	115	2.20	2.28*	86.34	−9.45	95.79
Glutamic acid	3.01	120	1.11	1.54	5.19	4.72	0.47
Cysteine	2.69	125	3.17	3.74	3.13	3.03	0.1
O-Methyl-tyrosine	1.13	165	2.49	3.12	8.05	7.01	1.04

* Ratio L-/D-Asp

bound AAs are faster than that of free amino acids. The extent of racemization during hydrolysis is difficult to predict. However, in the bound state the rate of inversion may differ for each individual amino acid residue and it is influenced by its neighbours (Frank, 1987). Standards of free amino acids present in Carbetocin (Cys, Ile, Leu, MeOTyr, Pro, Asp and Glu, the latter being hydrolysis products of Asn and Gln) were subjected to hydrolytic condition in our trials, separately in L- and D-forms. The course of racemization during hydrolysis from D- to L-AA form appeared to be very similar to the racemization from D- to L-amino acids (data not presented). Any racemization was not detected for Ile; the data for all AAs except of Gly and Pro are denoted in Table 1. MeOTyr was partially decomposed to Tyr during hydrolysis. We assume that the course of racemization of all L-AAs from L-Asn Carbetocin does not differ from that of D-Asn-Carbetocin.

Chiral determination of D,L-AAs

Resolutions and D/L ratios of free AAs measured isothermally are stated in Table 1. The resolution was calculated according to the formula:

$$\text{Resolution} = \frac{\text{RT}_\text{i} - \text{RT}_{\text{i}-1}}{0.5(\text{W}_{\text{i}-1} + \text{W}_\text{i})}$$

$$(\text{RT} = \text{retention time},\ \text{W} = \text{width of peak})$$

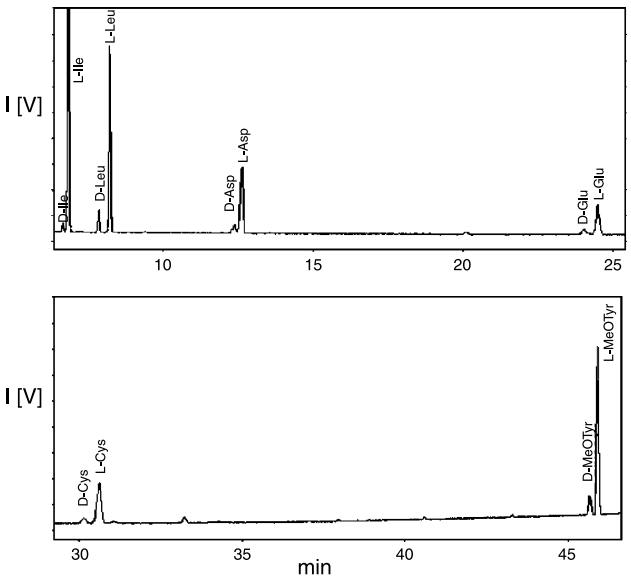


Fig. 1. GC/FID chromatograms of AA enantiomers as *N,O*-pentafluoropropoxycarbonyl pentafluoropropyl esters representing the ratio of D:L-AAs = 10:90 (2 nmol D-AAs:18 nmol L-AAs were derivatized) resolved on Chirasil-L-Val. For chromatographic conditions see Materials and methods

Resolution of tested AAs ranged from 1.13 (MeOTyr) to 3.78 (Leu). Chiral separation of AAs standards as *N,O*-pentafluoropropoxycarbonyl pentafluoropropyl esters (D to L-forms mixed at 1:9 ratios) is documented in Fig. 1. GC-FID chromatograms of PFPCF treated amino acids from hydrolysates of D-Asn Carbetocin and L-Asn Carbetocin, respectively, are shown in Figs. 2 and 3.

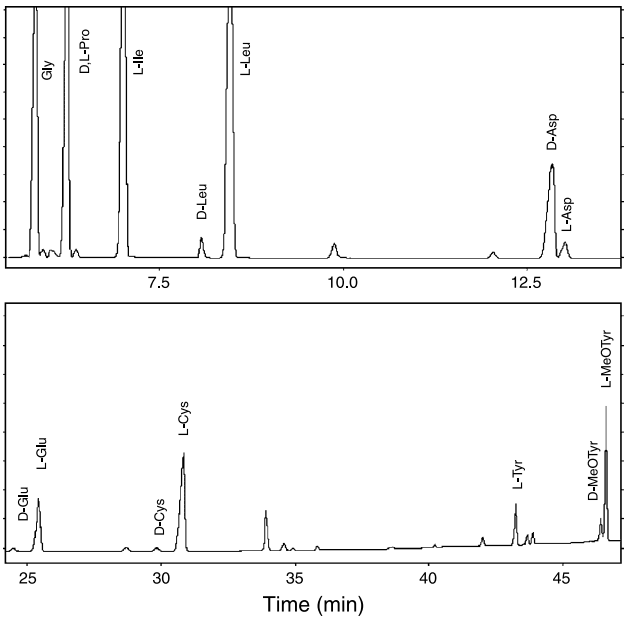


Fig. 2. GC/FID chromatograms of PFPC treated amino acids in D-Asn-Carbetocin (100 nmol) after hydrolysis separated on Chirasil-L-Val. For chromatographic conditions see Materials and methods

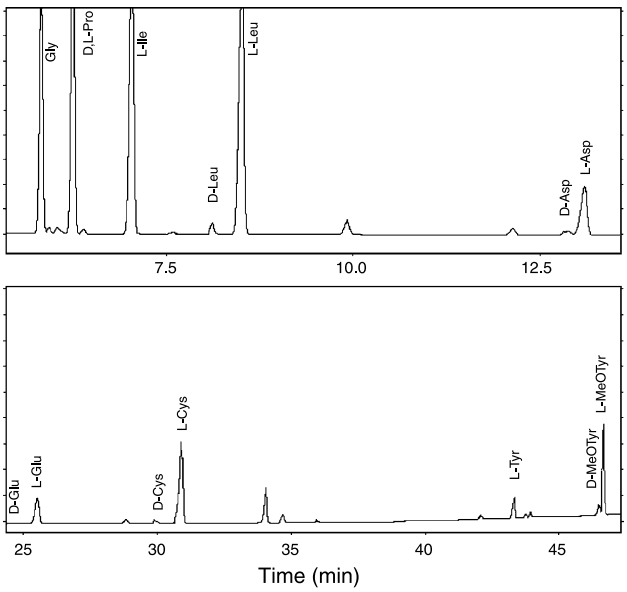


Fig. 3. GC/FID chromatograms of PFPC treated amino acids in L-Asn-Carbetocin (100 nmol) after hydrolysis separated on Chirasil-L-Val. For chromatographic conditions see Materials and methods

Table 2. Limit of detection obtained for *N*,*O*-pentafluoropropoxycarbonyl pentafluoropropyl esters of D,L-amino acids standards ($n = 6$, signal to noise ratio 3:1)

Amino acid	LOD (nmol/ml)
D-Leu	0.36
L-Leu	0.33
D-Asp	1.01
L-Asp	0.98
D-Glu	2.57
L-Glu	2.52
D-Cys	1.70
L-Cys	1.65
D-MeOTyr	0.44
L-MeOTyr	0.39

Enantiomeric purity of amino acids in D-Asn Carbetocin (net) was calculated by the following way: values obtained from racemization of L-Asn-Carbetocin due to hydrolysis were subtracted from those obtained from racemization of D-Asn-Carbetocin (racemization gross; Table 1). Due to the racemization the amount of D-Asn present in D-Asn Carbetocin is decreasing during hydrolysis that is why the value corresponding to racemization of Asp caused by hydrolysis has in Table 1 negative sign. The determination of enantiomeric purity of Pro was not possible as the enantiomers were not separated on the column.

The limit of detection (LOD in nmol/ml, signal to noise ratio 3:1) was determinated for the PFPCF derivatives (Table 2).

Conclusion

A novel sample preparation scheme has been developed for GC analysis of amino acid enantiomers in peptides. The procedure involves acid hydrolysis, followed by one step derivatization with a novel fluoroalkyl chloroformate. The reaction is running readily in aqueous media at room temperature. The novel derivatives of most amino acids are sufficiently volatile and easily separated on a chiral capillary column with a high rate of resolution. Relative standard deviations (RSD) of D/L-AAs ratios ranged from 1.11 (Glu in L-Asn-Carbetocin) to 3.74% (Cys in D-Asn-Carbetocin). The method proved to be suitable for rapid determination of optical purity of peptides of pharmaceutical importance.

Acknowledgement

The study was supported by Grant Agency of the Czech Republic, research project No. 203/04/0192 proceeding in the years 2004–6 and No. 303/06/1674, which is highly acknowledged.

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