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# Determination of the optical purity of threonine and hydroxyproline by capillary gas chromatography on a chiral stationary phase

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**Summary.** Experimental conditions for the derivatization and resolution by GLC of all stereoisomers of threonine and 4-hydroxyproline are reported. Threonine was in two steps converted to *N,O*-bisisobutoxycarbonyl 2,2,2-trifluoroethyl ester derivatives, the second of which was performed under anhydrous conditions. As such the enantiomers could pairwise be separated by capillary gas chromatography on a Chirasil-Val column. Since *L*- and *D*-threonine eluted much earlier than the corresponding allo forms, quantitative determination of the allothreonine content in *D*- or *L*-threonine down to the one percent level could be simply accomplished but also enantiomeric impurities could be determined. Unlike for threonine, the corresponding 4-hydroxyproline isomers could not all be resolved as *N,O*-bis-isobutoxycarbonyl 2,2,2-trifluoroethyl esters on this column. Although diastereomers could still be separated, the allo pair cochromatographed and the resolution for the *L*- and *D*-isomers was low. Complete separation of the 4-hydroxyproline isomers could be accomplished as *N,O*-bisprotected isobutyl amides, the formation of which required three derivatization steps. These were used for the determination of allohydroxyproline.

**Keywords:** Amino acids – Allohydroxyproline – Allothreonine – Chiral separation – Chirasil-Val – Hydroxyproline – Threonine

## Introduction

Gas chromatography is nowadays a well-established technique for the analysis of stereoisomers of various chemical compounds including amino acids (König, 1987). Introduction of chiral stationary phases (Gil-Av et al., 1966; Nakaparksin et al., 1970; Koenig et al., 1970), improvements in the preparation of capillary columns and derivatization techniques (Frank et al., 1977) were important milestones in this context. As an example, after acylation of their amino groups with pentafluoropropionic acid, 14 pairs of proteinogenic

amino acids could *simultaneously* be satisfactorily resolved as isopropyl esters between 90 and 170°C on a chiral polysiloxane column (Frank et al., 1977). Later other derivatives with improved properties were reported such as carbamates (Abe et al., 1995a), fluorinated (Husek, 1991) and other esters (Husek, 1998) and amides (Abe et al., 1995b). As a result more recently also *D,L*-proline and all four isomers of isoleucine could be easily resolved after introduction of *N*-isobutoxycarbonyl (*i*-Boc) groups and conversion of esters to isobutylamides (Abe et al., 1995b).

We have for some time prepared proteinogenic amino acids labelled with stable isotopes for application in peptide synthesis. This work involved asymmetric synthesis with chiral auxiliaries and in this context we set up and examined various analytical methods including gas chromatography on chiral columns for the determination of the optical purity of the products (Elemes and Ragnarsson, 1996). Among amino acids studied in this context was threonine, for which all four stereoisomers could be resolved as *N,O*-bisprotected 2,2,2-trifluoroethyl esters (Fransson and Ragnarsson, 1997). This work involved certain modifications in methodology in order to allow *O*-protection to take place. In this paper we describe the optimization of the procedure and demonstrate its application for determination of optical purity. Similar work has now also been undertaken with respect to the amino acid hydroxyproline, which although not belonging to the coded proteinogenic amino acids occurs in collagen, the major protein of connective tissue. To our knowledge mixtures of hydroxyproline isomers have not previously been completely resolved by gas chromatography.

## Materials and methods

### *Equipment*

The gas chromatograph was a Shimadzu model GC-14A (Shimadzu, Kyoto, Japan), equipped with a hydrogen FID and an automatic injector (AOC-14). It was used together with Axxi-Chrom™ 727 computer software (Axxiom Chromatography, Inc., Calabasas, CA) and a Chirasil-*D*-Val column, 25 m x 0.25 mm, film thickness 0.11 $\mu$m (Chrompack, Middelburg, The Netherlands). To improve mixing during derivatization, reaction vials were kept in an ultrasonic bath, 35 kHz, 120–240 W (type RK 106; Bandelin, Berlin, Germany).

### *Materials*

The following amino acids were used in this study: *L*-threonine (Tanabe), *D*-threonine (General Biochemicals, Chagrin Falls, OH), *L*- and *D*-allothreonine (Bachem, Bubendorf, Switzerland), *L*-hydroxyproline (Sigma), *D*-hydroxy- and *L*-allohydroxyproline (gifts from Bachem) and *D*-allohydroxyproline (Sigma).

Other chemicals were as follows: 2,2,2-Trifluoroethanol for synthesis (Merck), isobutyl chloroformate and isobutylamine (Fluka), dichloromethane and dry pyridine for analysis (both Merck). The pyridine should *not* be further dried over molecular sieves.

### *Methods*

Threonine was converted to *N,O*-bis-*i*-Boc threonine 2,2,2-trifluoroethyl ester as follows: $A_1$) To a 0.05 M solution of threonine (50 $\mu$l), was first pipetted 2,2,2-

trifluoroethanol/pyridine (3:1; 100 $\mu$l) and then *i*-Boc-Cl (25 $\mu$l) added with a syringe, whereupon the vial was kept for 5 min in an ultrasonic bath. After the reaction mixture had been taken to dryness in a stream of nitrogen, the sample was dried for 10 min in vacuo below 0.1 mm Hg to remove traces of *i*-butanol formed and then dissolved in dry pyridine (200 $\mu$l). $B_1$) *i*-Boc-Cl (50 $\mu$l) was again added with a syringe and sonicated for 5 min, whereupon the pyridine was removed in a gentle stream of nitrogen before the residue was dissolved in *dichloromethane* (instead of diethyl ether, which avoids precipitation of pyridine hydrochloride and results in a higher and more reproducible recovery). 0.5–1.0 $\mu$l of this solution was injected onto the Chirasil-*D*-Val column. Remaining small amounts of pyridine normally requires about 15 min to elute, allowing a good baseline to be established.

Hydroxyproline was converted to *N,O*-bis-*i*-Boc hydroxyproline *i*-butylamide as follows: $A_2$) This step was performed as described under $A_1$ above for threonine including drying in vacuo. $B_2$) Modified Abe procedure (Abe et al., 1995b): The residue was suspended in isobutylamine (300 $\mu$l) and the vial sonicated for 5 min, whereupon the excess amine was carefully removed in a gentle stream of nitrogen. The residue was dissolved in dry pyridine (200 $\mu$l). $C_2$) *i*-Boc-Cl (100 $\mu$l) was added, whereupon the sample was treated as under $B_1$.

## Results

### *Optimization experiments for preparation of the required N,O-disubstituted derivatives*

As a first step towards the analysis of threonine isomer mixtures based on formation of *N,O*-protected esters (Fransson and Ragnarsson, 1997), the amount of *i*-Boc-Cl required to derivatize the threonine OH was determined. These experiments were performed in pyridine and the results are presented in Fig. 1. Based on these data the amount of *i*-Boc-Cl used in step $B_1$ under *Methods* above could be reduced to 50 $\mu$l from 100 $\mu$l applied in our preliminary work without noticeable effect (Fransson and Ragnarsson, 1997). For hydroxyproline in step $C_2$ 100 $\mu$l reagent seemed to give better results and was therefore used.

### *Application to determination of optical purity of threonine*

A full chromatogram demonstrating the resolution of all four isomers of threonine was shown in our previous communication (Fransson and Ragnarsson, 1997). Therefore, in this context we are going to restrict the presentation with respect to threonine to two quantitative applications, both illustrating determination of optical purity of threonine. Figure 2 demonstrates results from experiments with *L*-threonine, spiked with decreasing amounts of *L*-allothreonine, after derivatization in two steps as discussed above. When applied to our old sample of this amino acid, we could show that it contained 0.95% of the *L*-allo isomer.

Similarly, *L*-threonine was spiked with decreasing amounts of *D*-threonine and derivatized. The results are shown in Fig. 3. In this case the modest resolution of the peaks did not allow application of a large amount of sample without loss of resolution.

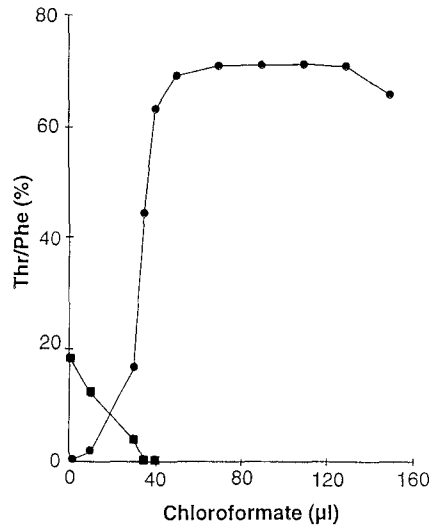


**Fig. 1.** Optimization experiment. An equimolar threonine/phenylalanine mixture was derivatized as described under *Methods* above with increasing amounts of *i*-Boc-Cl in step $B_1$ and chromatographed at 140°C on a Chirasil-*D*-Val column. The ratio between the peak areas for the trifluoroethyl esters of *N,O-i*-$Boc_2$-Thr (II) and *N-i*-Boc-Phe (III) was plotted (●). At low amounts of *i*-Boc-Cl, remaining *N-i*-Boc-Thr ester (I) was also visible. The ratio I/II was plotted (■). $t_{RI}$ = 20.6 min; $t_{RII}$ = 24.8 min; $t_{RIII}$ = 31.9 min

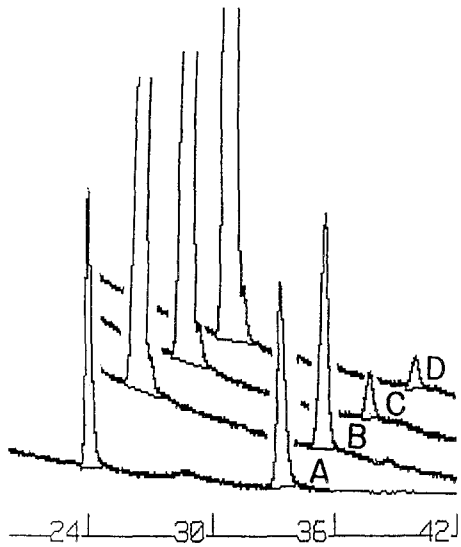


**Fig. 2.** *L*-Threonine/*L*-allothreonine mixtures, chromatographed on a Chirasil-*D*-Val column at 140°C. (**A**) 50:50; (**B**) 9:1; (**C**) 99:1; (**D**) no *L*-allothreonine added, indicating the presence of 0.95% of this isomer as impurity. Injected amount (**B–D**) ~ 1 µg

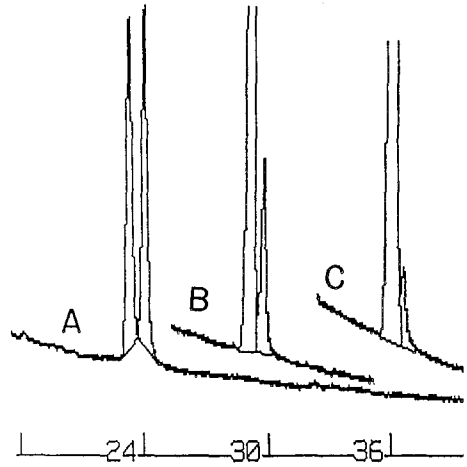


**Fig. 3.** *L*-Threonine/*D*-threonine mixtures, chromatographed on a Chirasil-*D*-Val column at 140°C. (**A**) 50:50; (**B**) 9:1; (**C**) 99:1

*Hydroxyproline*

Isomers of hydroxyproline could not be completely separated as *N,O*-protected trifluoroethyl esters. In this case the allohydroxyproline esters cochromatographed and the resolution for the *L*- and *D*-isomers was low ($t_R$ 32.1 min for *L*- and *D*-allohydroxyproline; 96.3 min for *L*-hydroxyproline; 99.1 min for *D*-hydroxyproline/155°C). Hydroxyproline was therefore instead converted to isobutylamide and chromatographed after *O*-protection as described under Methods above. The results are shown in Fig. 4.

The procedure described in Fig. 4 can be applied to determine the amount of *L*-allohydroxyproline present in *L*-hydroxyproline at least down to the 1% level as shown in Fig. 5.

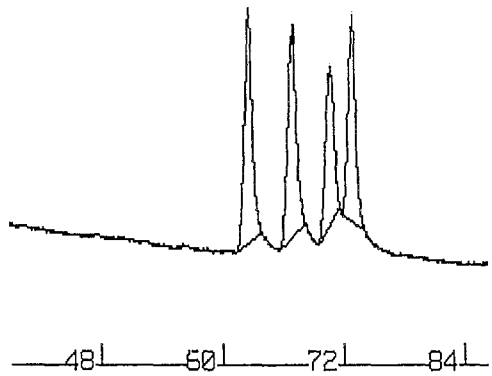


**Fig. 4.** Equimolar hydroxyproline mixture, chromatographed as *N,O*-bis-isobutyloxycarbonyl isobutyl amides on a Chirasil-*D*-Val column at 180°C. The peaks appear in the following order: *L*-Hydroxyproline ($\alpha$ = 1.00), *L*-allohydroxyproline ($\alpha$ = 1.07), *D*-allohydroxyproline ($\alpha$ = 1.13) and *D*-hydroxyproline ($\alpha$ = 1.17)

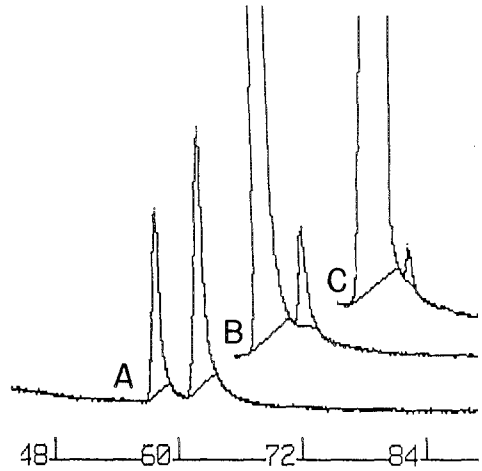


**Fig. 5.** Determination of *L*-allohydroxyproline present in *L*-hydroxyproline after conversion to *N,O*-bisisobutyloxycarbonyl isobutyl amides with separation on a Chirasil-*D*-Val column at 180°C. (**A**) 50:50; (**B**) 9:1; (**C**) 99:1

## Discussion

The key to the successful chromatographic resolution of threonine and hydroxyproline as reported above was the introduction of *i*-Boc groups on their hydroxyl groups. In the reaction between a hydroxy amino acid such as threonine with *i*-Boc-Cl in pyridine/trifluoroethanol medium (Abe et al., 1995a,b) also containing water, fast simultaneous derivatization of both the amino and carboxyl groups takes place as first shown by Husek for a closely related system (Husek, 1991). No reaction occurs between the chloroformate and the hydroxy group in this step as seen in Fig. 1. Its nucleophilicity is low in comparison to that of the amino and the carboxyl groups and even after the latter have reacted, the hydroxyls can not compete with the trifluoroethanol and water present in large excess. Therefore, in order to make them react, we carried out a second chloroformate reaction in the *absence* of competing alcohol and water. Neat, dry pyridine is a suitable solvent for this reaction. This step was carefully optimized. In some experiments we used pyridine dried over molecular sieves (4A) and got rather confusing results. Later we found out that 2–3 times more *i*-Boc-Cl was required when such pyridine was applied, for which fact at this stage no explanation can be given.

The exceptional separation of threonine diastereomers after derivatization in this way (Fransson and Ragnarsson, 1997) indicated that it would be practically useful for determination of small amounts of allothreonine in threonine or threonine in allothreonine samples. This was illustrated for the *L*-threonine samples spiked with *L*-allothreonine above (Fig. 2), from which we conclude that, with access to an integrator, accurate such determinations can be performed below the 1% level. On the other hand, the more modest separation of the enantiomers in this case made it less

suitable for the characterization of such mixtures as can be seen from Fig. 3, in which *L*- and *D*-threonine in different ratios were chromatographed.

As briefly indicated above, after an identical derivatization diastereomeric derivatives of hydroxyproline were also well separated on the Chirasil-Val column, whereas the enantiomeric ones were not. For this 4-hydroxy amino acid, the unresolved allo pair has the shortest retention time, contrary to what is the case for threonine with its 3-hydroxy group. Because of the poor resolution among the enantiomers, we chose to investigate the isobutylamides instead of trifluoroethyl esters of hydroxyproline. Such derivatives were recently introduced by Abe and coworkers who found them superior with respect to resolution of proline (Abe et al., 1995b). Due to the sensitivity of *O*-*i*-Boc-derivatives to nucleophiles, the aminolysis step in this case must be performed prior to *O*-protection. Thus for hydroxyproline a somewhat lengthy three-step derivatization procedure was required but it payed off in nice resolution of all four isomers as shown in Fig. 4. This procedure was applied to mixtures of *L*-hydroxy- and *L*-allohydroxyproline and it proved to be useful for determination of the latter isomer down to the 1% level (Fig. 5).

In summary, we have developed procedures allowing *simultaneous* separation of all the four stereoisomers of threonine or hydroxyproline on a Chirasil-Val column. Such procedures should have applications in analytical biochemistry. They also make it possible to monitor the optical purity in synthetic work (Lloyd-Williams et al., 1997) including isotope-labelling (Sutherland and Willis, 1997).

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