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Efficient enantioselective separation and determination of trace impurities in secondary amino acids (*i.e.*, imino acids)

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ABSTRACT

An *R*-(−)-1-(1-naphthyl)ethyl carbamoylated-β-cyclodextrin bonded phase in conjunction with a nonaqueous polar mobile phase was used for the highly selective enantioseparation of a number of secondary amino acids after their pre-column derivatization with 9-fluorenylmethyl chloroformate (FMOC). Under the conditions employed, the FMOC reagent served to "lock" the imino acid into their existing conformation thereby preventing the possibility of racemization. Furthermore, it served to increase the sensitivity to the point that trace level enantiomeric impurities were easily detected. Compared with separations that use traditional reversed-phase solvents, this method showed several advantages: higher selectivity towards the imino acid enantiomers investigated, shorter analysis times, faster equilibration of the column, more stable baseline and more sensitive fluorescence detection. The detection limits for FMOC derivatives of proline, *trans*-4-hydroxyproline, *cis*-4-hydroxyproline, pyroglutamic acid, 3,4-dehydroproline, thiaproline, penicillamine acetone adduct and pipecolic acid are in the low femtomole range. The method was used for evaluation of enantioselectivity of a number of "optically pure" commercial imino acid standards. Enantiomeric impurities as low as 0.0001% (1 ppm) can be determined in some cases. High precision determination of trace levels of D-imino acids in the presence of large amounts of corresponding (opposite) L enantiomer at 1, 0.1, 0.01% and below are demonstrated.

INTRODUCTION

Amino acids form a large group of compounds of pharmaceutical and biochemical interest. The stereoisomers of amino acids differ in biological activity, hence their configuration and optical purity are very important in many fields of peptide and polypeptide chemistry. Because D-amino acids are rare in nature compared with the L enantiomers, their determination in complex biological samples requires selective, efficient and highly sensitive screening techniques. As has been shown in recent years, the method of choice often is high-performance liquid chromatography (HPLC) with fluorimetric detection.

In the past two decades, a number of different HPLC procedures have been developed for enantiomeric separation of amino acids using both direct and indirect methods. The progress made in the field can be found in many original research papers and review articles and will not be discussed in detail here [1–3].

HPLC methods are often combined with pre- or post-column derivatization in order to achieve high-sensitivity fluorimetric or photometric detection. Many chromatographic procedures use derivatization reagents such as 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) [4–6], dansyl chloride (DNS-Cl) [7–12], phenylisothiocyanate (PITC) [13–15], *ortho*-phthalaldehyde–mercaptoethanol (OPA–

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ME) [16–20], 9-fluorenylmethyl chloroformate (FMOC-Cl) [21–25] and naphthalenedialdehyde and analogues [26,27]

The above methods have been successfully used for chiral and achiral separation of a number of amino acids However, each method has distinct advantages and disadvantages It is especially difficult to conduct the DNS-Cl derivatization reaction adequately when the amino acids are present in low concentration in complex mixtures [28] The OPA–ME derivatization is more rapid and sensitive, but its use is limited to primary amino acids The PITC reagent requires an evaporation of the sample to remove excess of reagent prior to HPLC In addition, all of these derivatives show only limited stability The labeling with FMOC-Cl avoids the problems mentioned above and combines high sensitivity in fluorescence detection with favorable chromatographic properties and ease of derivatization for both primary and secondary amino acids

This paper presents the results of enantioseparation of a number of secondary amino acids (imino acids) including naturally occurring proline, hydroxyprolines, pipecolic acid and pyroglutamic acid Their physiological and pathological roles have received attention from numerous workers [29–31] Several different HPLC techniques have been used for quantitative achiral determination of secondary amino acids in biological samples [5,22, 25,32–38], however a precise, accurate and sensitive method for chiral determination is still needed

The enantiomeric separation of the FMOC–imino acids was obtained on a *R*-(−)-1-(-naphthyl)ethyl carbamoylated-β-cyclodextrin (RN-β-CD) column which has been previously used for successful resolution of a large variety of enantiomers in the normal-phase and reversed-phase mode [22,39,40] In the present study the RN-β-CD column, operated with a nonaqueous polar mobile phase, was used for separation of secondary amino acids after their pre-column derivatization with FMOC-Cl To our knowledge, it is the first enantioseparation of highly fluorescent FMOC derivatives of proline [22], pipecolic acid and their analogues The method was used for determination of enantiomeric contamination of a number of "optically pure" commercial imino acid standards

EXPERIMENTAL

Apparatus

The HPLC system consisted of three pumps (LC-6A, Shimadzu, Kyoto, Japan), a system controller (SCL-6B, Shimadzu), UV detector (SPD-6A, Shimadzu), fluorescence detector (RF-535, Shimadzu), switching valve (Rheodyne, Cotati, CA, USA) and 0 2-μl injector valve (Valco, Houston, TX, USA) The columns were 100 × 4 6 mm C_{18}, 250 × 4 6 mm acetylated-β-cyclodextrin (AC-β-CD) and RN-β-CD (Astec, Whippany, NJ, USA)

Chemicals

Amino acids were purchased from different sources listed in Table II Acetonitrile, water, acetic acid and triethylamine were of HPLC grade and supplied from EM Science (Gibbstown, NJ, USA)

Procedure

Derivatizing agent FMOC-Cl was purchased from Sigma (St Louis, MO, USA) Derivatization was performed according to ref 21 D-Thiaproline was obtained by racemization of L-thiaproline in boiling 6 *M* HCl solution *trans*-4-Hydroxy-D-proline was obtained by epimerization of *cis*-4-hydroxy-L-proline according to ref 41 Isomerization degree of both racemization and epimerization reactions was checked using a C_{18}/RN-β-CD column switching method After derivatization of post reaction mixtures with FMOC-Cl, 5 μl of the sample was injected onto the C_{18} column and chromatographed using acetonitrile–water–acetic acid (200 800 2, v/v/v) at 0 5 ml/min A UV wavelength of 266 nm was used to monitor the effluent The switching valve was turned for 2 s after the signal reached the maximum of the standard retention time In this way a small portion of the eluting peak of *trans*-4-hydroxyproline or thiaproline was introduced into the chiral column

RESULTS AND DISCUSSION

Optimization of mobile phase composition

Most FMOC-functionalized imino acids can be resolved in either a conventional reversed-phase mode (hydro-organic solvents) or with a mobile phase consisting almost entirely of acetonitrile (containing small amount of glacial acetic acid and triethylamine modifiers) The latter of these will be

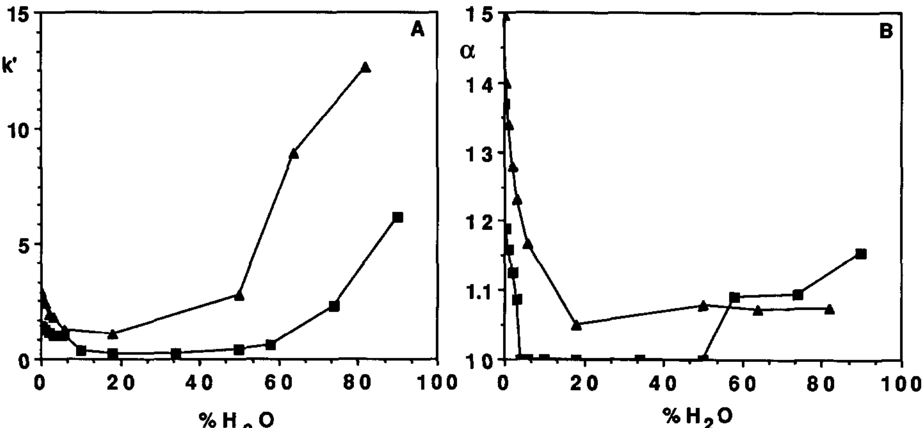

Fig 1 Influence of water concentration in the mobile phase on (A) the retention and (B) enantioselectivity obtained on AC-β-CD (■) and RN-β-CD (▲) column for D, L-proline Eluent acetonitrile–water–0 6% triethylamine–0 4% acetic acid Flow-rate 1 ml/min

referred to as the "polar organic mobile phase"

As can be seen in Figs 1 and 2, for proline, separations done with polar organic mobile phase are generally preferable to those done with a hydro-organic mobile phase This is because the enantioselectivity (α) is significantly greater with a polar organic mobile phase, while retention times are less Fig 1 also shows that the *R*-(−)-1-(1-naphthyl)-ethyl carbamoylated-β-cyclodextrin column is generally more selective for the FMOC–imino acids than is the acetylated-β-CD column It is also interesting to note that in the reversed-phase mode, the large increases in retention with mobile phases containing more than 50% buffer did not produce corresponding improvements in the separation or the enantioselectivity (Fig 1)

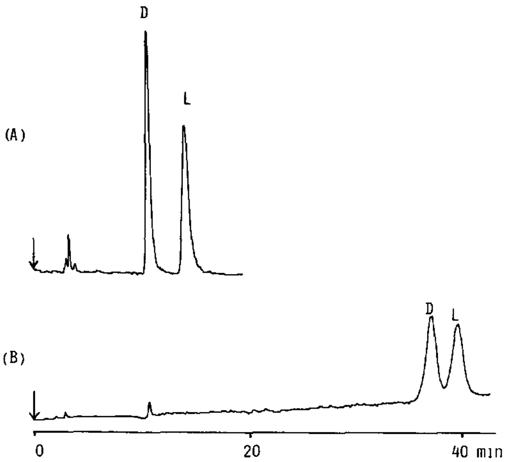

Fig 2 Enantioseparation of D, L-proline obtained on RN-β-CD column in (A) nonaqueous system, eluent acetonitrile–triethylamine–acetic acid (1000 6 4), (B) under optimal conditions in aqueous system Eluent water–acetonitrile–triethylamine–acetic acid (850 150 6 4) Flow-rate 1 ml/min The time scale at the bottom of the figure applies to both chromatograms (A) and (B)

In the case of the acetylated-β-CD stationary phase, the addition of as little as 4% (v/v) water to the mobile phase negated the enantioselectivity (Fig 1B) These results indicate that the chiral recognition mechanism may be dependent on the mobile phase composition In the case of 1-(1-naphthyl)ethyl carbamoylated-β-cyclodextrin columns, this phenomenon has been previously reported and discussed in detail [12,39,40] The aforementioned conclusion is also supported by the results shown in Fig 3 The addition of water into an acetonitrile mobile phase influences not only the selectivity, but also efficiency of the column The plots shown in Fig 3 can give some information on the molecular recognition mechanism Following the general non-equilibrium theory developed by Giddings [42], the overall plate height (H) for liquid–solid chromatography (LSC) can be expressed as

$$H = A + \frac{B}{u} + C_m\bar{u} + C_k\bar{u} \quad (1)$$

Zone spreading is due to three independent

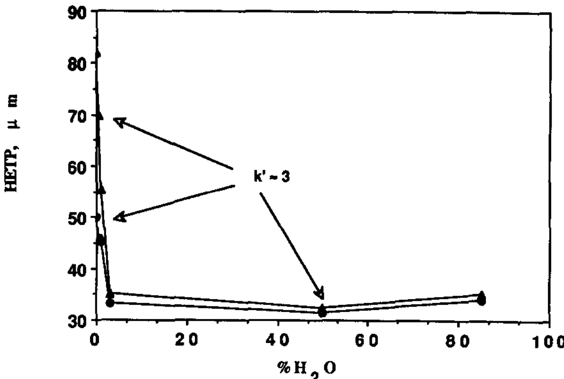

Fig 3 Influence of water concentration in the mobile phase on efficiency of RN-β-CD column, ▲ = L-proline, ● = D-proline Chromatographic conditions as in Fig 1

sources flow pattern effects (A), longitudinal diffusion (B) and mass transfer effects (C) Flow pattern effects depend on the structure of the porous support material and are independent of the eluent velocity (u) Ordinary molecular diffusion in the flow direction contributes most at low velocities and in LC the term B/u is small Mass transfer effects contribute most in high-speed runs In adsorption chromatography they are controlled by two basically different mechanisms or some combination thereof diffusion-controlled sorption and desorption rates originating in the mobile phase (C_m) and adsorption–desorption kinetics (C_k)

For the same chromatographic system, the contribution of the first three terms to H is the same for both enantiomers

According to

$$C_k = \frac{2q}{k_d} \frac{k'}{(1 + k')^2} \tag{2}$$

where q is the geometrical parameter, k_d is the desorption rate and k' is the capacity factor, the significant differences in column efficiency observed for proline enantiomers in the non-aqueous system indicates that there is a considerable difference in adsorption–desorption kinetics for both enantiomers Indeed, for the stronger retarded L-enantiomer, the rate of the adsorption–desorption process is about 2 times lower than for the D enantiomer

The change in the eluent composition influences the mass-transfer effects controlled by diffusion in the mobile phase

Since

$$C = \frac{wd_p^2}{D_m} \tag{3}$$

where w is a dimensionless constant, d_p is the particle size and D_m is the diffusion coefficient in the mobile phase The change in the eluent composition also influences the diffusion velocity because of changes in the solvent viscosity ($D_m \approx 1/\eta$, where η = viscosity) However, the dramatic decrease of the height equivalent to a theoretical plate (HETP) values for both proline enantiomers cannot be attributed to the mass transfer effects in the mobile phase The effect observed is just opposite to that which should be expected from theory the addition of water induces a large increase in the solvent viscosity which should also increase the contribution of C_m term of the plate height Moreover, the addition of water significantly decreases capacity factors in the discussed region [0–4% (v/v) water] Because $k'/(1 + k')^2$ (eqn 2) is an increasing function with decreasing k' values ($k' > 1$), the reduction in the HETP values also cannot be due to changes in the capacity factors Despite a large increase in solvent viscosity [η (water) = 1 cP, η (acetonitrile) = 0 34 cP] in the true reversed-phase mode, the plate heights for both enantiomers are similar, and only slightly dependent on eluent composition in the range studied

Interpretation of the data leads to the assumption that the addition of water to the eluent changes the chiral recognition mechanism and influences the kinetic process of sorption–desorption in the stationary phase which results in narrower peaks The HETP values obtained in aqueous systems are lower (for the similar capacity factor as indicated in Fig 3) compared with HETP found in the nonaqueous system The results suggest the existence of at least two types of recognition mechanisms which differ in the rate of adsorption–desorption process

It should be mentioned that in contrast to traditional aqueous systems, the recognition mechanism in nonaqueous systems is still not clear It has been postulated that an inclusion complexation may not be occurring in these conditions [43] Rather, a more external adsorption at the mouth of the cyclodextrin cavity could account for the observed separations

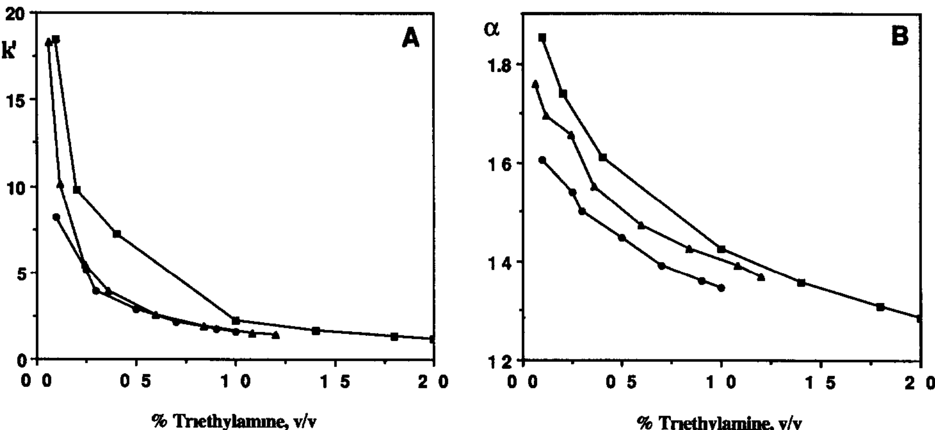

Fig 4 Dependence of (A) the retention and (B) enantioselectivity on total amount of triethylamine and acetic acid and their relative ratio added to neat acetonitrile Test compound D, L-proline Column RN-β-CD Eluent acetonitrile–triethylamine–acetic acid Triethylamine acetic acid ■ = 2 1, ● = 2 3, ▲ = 1 2

including the apparent size selectivity between α, β and γ-cyclodextrin [43]

Fig 2 shows the enantioseparation of a proline racemate obtained on RN-β-CD column in the nonaqueous mode and under optimal conditions in a water-rich system The preliminary investigations have shown that the water-free system has several advantages over the aqueous one including greater resolution, faster equilibration of the column, more stable base-line and lower detection limits

Thus, the RN-β-CD column which exhibited high selectivity toward FMOC–D, L-proline, and a non-aqueous eluent was chosen for the detailed study on the optimization of enantioseparation of secondary amino acids

As indicated in Fig 4, retention and selectivity of nonaqueous systems can be effectively regulated by changes in two parameters of the mobile phase the total amount of triethylamine and acetic acid added and their relative ratios Fig 4 presents the typical dependence for all imino acids investigated This behavior may be used for an optimization of separation factors for any analogous separation problem Fig 5 shows the influence of triethylamine and acetic acid concentration on the enantioseparation of pipecolic acid racemate

Selectivity and sensitivity of the method

The chromatographic data for all racemic mixtures investigated are summarized in Table I The selectivity of the system was regulated by adjusting the mobile phase composition to achieve base-line resolution with a reasonable retention time The combination of good selectivity ($\approx$1 15–1 50) and high efficiency results in high resolution factors for FMOC proline enantiomers and its analogues

The enantioseparation of pipecolic acid was achieved with an eluent containing lower concentrations of triethylamine and acetic acid, which resulted in a longer analysis time The separation of nipecotic acid (an analogue of pipecolic acid) was not possible

It should be mentioned that high selectivities and resolutions listed in Table I are more than adequate

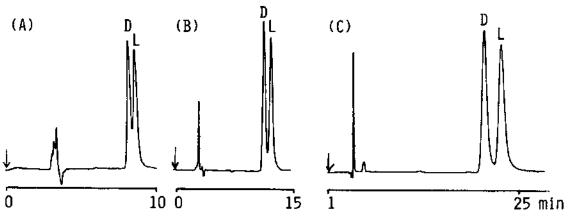

Fig 5 Effect of total amount of triethylamine and acetic acid per 1000 ml of acetonitrile in the mobile phase on the resolution of D, L-pipecolic acid obtained on RN-β-CD column Flow-rate 1 ml/min Eluent (A) acetonitrile–0 6% triethylamine–0 9% acetic acid, (B) acetonitrile–0 3% triethylamine–0 45% acetic acid, (C) acetonitrile–0 12% triethylamine–0 18% acetic acid

TABLE I

SEPARATION DATA FOR RACEMIC MIXTURES OF SECONDARY AMINO ACIDS ON RN-β-CD COLUMN OPERATED WITH A NONAQUEOUS MOBILE PHASE

Name	Structure	Eluent[a]	k'_D	k'_L	α	R_s[c]
Proline		6 4 1000	2 9	4 4	1 5	4 1
trans-4-Hydroxyproline		6 4 1000	7 3	9 6	1 3	2 3
cis-4-Hydroxyproline		6 4 1000	1 8	2 2	1 2	1 7
		3 2 1000	3 2	4 1	1 3	2 7
Pyroglutamic acid[b]		6 4 1000	1 9	2 4	1 3	3 3
3,4-Dehydroproline		6 4 1000	2 6	3 6	1 4	3 5
Thiaproline		6 4 1000	1 9	2 2	1 2	2 6
Penicillamine acetone adduct		6 4 1000	1 2	1 4	1 2	1 5
		3 2 1000	1 9	2 3	1 2	2 0
Pipecolic acid		6 4 1000	2 2	2 3	1 1	1 1
		3 2 1000	3 5	3 8	1 1	1 4
		1 5 1 1000	6 2	6 9	1 1	1 7

[a] Triethylamine–acetic acid–acetonitrile

[b] Although this is an amide it is known to react with a variety of acid chlorides [49,50] In this work, we found that it reacts with FMOC-Cl as well

[c] R_s = Resolution

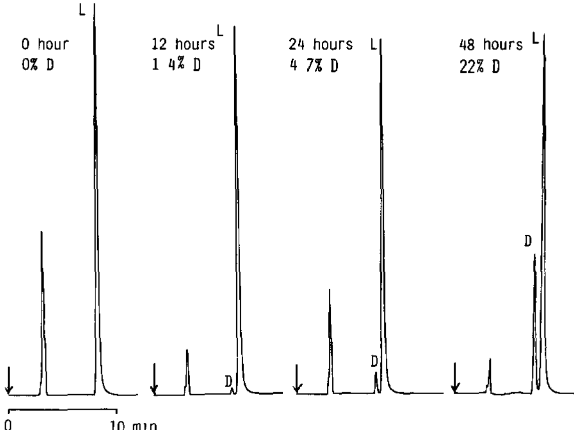

Fig 6 Change of enantiomeric ratio of thiaproline during the racemization reaction (see Experimental) Eluent acetonitrile–triethylamine–acetic acid (1000 6 4) Flow-rate 1 ml/min

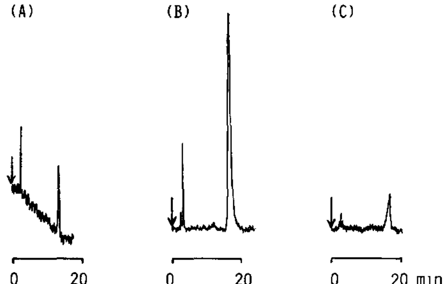

Fig 7 Comparison of detectability of L-proline in (A) aqueous eluent [water–acetonitrile–triethylamine–acetic acid (530 470 6 4)] and (B, C) nonaqueous eluent [acetonitrile–triethylamine–acetic acid (1000 6 4)] Column RN-β-CD Flow-rate 1 ml/min Injected amount (A) 7 4 fmol, (B) 7 4 fmol and (C) 1 4 fmol

for determinations of racemic mixtures However, as has recently been reported [44], the requirement for trace analysis are more rigorous Resolution factors greater than 2 for the racemic mixture is an appropriate criterion for quantitative trace analysis applications

The elution order was checked by the injection of pure enantiomers or mixtures with different enantiomeric ratios Since for thiaproline and *trans*-4-hydroxyproline, only L enantiomers were available, the enantiomers were prepared via racemization and epimerization reactions, respectively, according to the procedure described in the Experimental section The degree of racemization was checked by HPLC Fig 6 shows the change of ratio of thiaproline enantiomers during the racemization reaction

The D enantiomer was eluted prior to the L enantiomer for all the FMOC–imino acids examined, which is an advantage because the L enantiomer is the dominant component in most biological samples It has been shown that when traces are eluted before the major enantiomer the quantitative determination of chiral composition becomes more accurate and precise [45,46], and trace detectability can be improved tremendously [47]

The detection limits were calculated using a signal-to-noise ratio of 2 The detection limits for all enantiomers investigated are in the low femtomole range It was found that the sensitivity of the method depends on eluent composition The detection limit for FMOC L-proline, estimated in the nonaqueous system, was 5 times lower than was found for the system with a mobile phase containing 53% of water (see Fig 7) This is probably due to the fact that for many aromatic and/or heteroaromatic compounds water has a quenching effect on the fluorescence [48] In addition, as shown in Fig 7, the noise and baseline shifting is more prevalent in a water-rich system

Practical applications

This method was used for determination of enantiomeric contamination in a number of commercial imino acid standards The results are collected in Table II The chromatographic conditions are given in Table I Some level of enantiomeric contamination was found in all commercial amino acid samples As can be seen from the results in Table II the method enables trace and ultra-trace determination of optical purity for both L and D enantiomers

Quantitative analysis of contaminating D enantiomers in L-amino acids at 1, 0 1 and 0 01% gave very high precision (R S D for 4 measurements) of 0 3% (pyroglutamic acid), 2 6% (*cis*-4-hydroxyproline) and 4 5% (FMOC-L-proline), respectively

For the L enantiomer, as the trace component, the situation is more complicated The problem associated with tailing from the enantiomerically rich component results in quantitative determinations with lower precision This is shown in Fig 8B The precision in quantitative analysis for optical purity of proline enantiomers at the 0 1% level is 1 2% and

TABLE II

OPTICAL PURITY OF COMMERCIAL SAMPLES OF L- AND D-AMINO ACIDS

Chromatographic conditions as in Table I

Name	Source	Concentration of opposite enantiomer (%)	Eluent[a]	Standard deviation[d]
D-Proline	ICN	0 7	A	0 08
L-Proline	Aldrich	0 5		0 006
FMOC-L-proline	Fluka	0 02	A	0 0008
trans-4-Hydroxy-L-proline[b]	Sigma	0	A	
		0 09[b]		0 008
cis-4-Hydroxy-D-proline	Sigma	0 4	B	0 009
cis-4-Hydroxy-L-proline	Sigma	1 5	B	0 007
D-Pyroglutamic acid[c]	Sigma	2 5	A	0 04
L-Pyroglutamic acid[c]	Sigma	1 3	A	0 004
L-Thiaproline	Sigma	0 3	A	0 009
L-Penicillamine acetone adduct	Sigma	0 3	B	0 01
3,4-Dehydro-L-proline	Aldrich	0 4	A	0 04
L-Pipecolic acid	Aldrich	0 3	C	0 007

[a] Eluent acetonitrile–triethylamine–acetic acid A = 1000 6 4, B = 1000 3 2, C = 1000 1 5 1

[b] *cis*-4-Hydroxy-D-proline, according to [43], *trans*-4-hydroxy-L-proline epimerizes to *cis*-4-hydroxy-D-proline

[c] Although this is an amide it is known to react with a variety of acid chlorides [49,50] In this work, we found that it reacts with FMOC-Cl as well

[d] $n = 5$

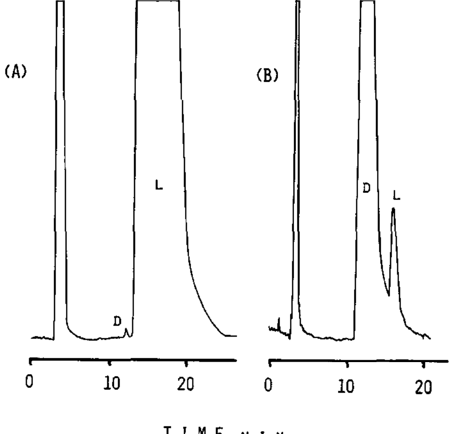

Fig 8 Chromatograms used to evaluate the enantiomeric purity of (A) L-proline (a purified mixture) and (B) D-proline (ICN) Eluent acetonitrile–triethylamine–acetic acid (1000 6 4) Column RN-β-CD Flow-rate 1 ml/min

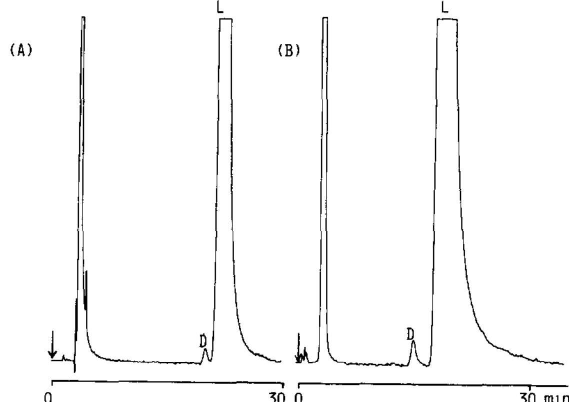

Fig 9 Chromatograms used to evaluate the enantiomeric purity of (A) L-pipecolic acid (Aldrich) Eluent acetonitrile–triethylamine–acetic acid (1000 1 5 1), (B) *cis*-4-hydroxy-L-proline (Sigma) Eluent acetonitrile–triethylamine–acetic acid (1000 3 2) The other conditions as in Fig 8

11 1% for the D and L enantiomer, respectively The trace analysis precision for the L enantiomer found in this study was 1 8% (pyroglutamic acid) at the 1% level and 11 1% (proline) at the 0 1% level

In conclusion, the present method gives high sensitivity and precision for the evaluation of trace levels of D imino acids in the presence of large amounts of the corresponding L enantiomer Fig 9 shows the chromatographic separation of contaminating D enantiomers in L-pipecolic acid and *cis*-4-hydroxy-L-proline samples

This technique can be easily adopted to routine analysis and has been used extensively in this laboratory to quantitate the D-proline content in physiological fluids down to the ppm level (Fig 8A)

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