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Racemization of L-Lysine for Pharmaceutical Synthesis and Its Chiral Separation by GC-MS Spectroscopy

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In order to improve physico-chemical properties and to enhance stability of drugs, amino acid salt has been widely adopted in pharmaceutical synthesis. Acetylsalicylic acid lysinate is one of the widely used analgesics and it is a good example of this synthesis. In the case of acetylsalicylic acid lysinate synthesis, racemization of naturally occurred lysine is essential because the racemic lysine salt of the drug shows better yield, crystallinity and dryness than that of the L-lysine salt. To establish a simple, practical and economical process for L-lysine racemization, L-lysine treatments with phosphoric acid and with acetic acid were compared and the optimum conditions for its process and derivatization were investigated by chiral separation methods using GC-MS spectroscopy.

Key words : Enantiomer, Acetylsalicylic acid, Lysine, Racemization, GC-MS, Chiral, TFA, PFPA

INTRODUCTION

Lysine is one of the amino acids which are found in nature as constituents of proteins. Natural lysine is optically active L-(+)-lysine, which is an essential component of animal diets whereas the enantiomeric D-(-)-lysine is known to have no nutritional value (Emmick *et al.*, 1952). Synthesized lysine consists of equal parts of the biologically active L-(+)-isomer and biologically inactive D-(-)-isomer. This synthesized form is commonly designated as a racemic mixture. It has been adopted to form the L-lysine salt of compounds, including ibuprofen (Reynolds *et al.*, 1991), bendazac, ketoprofen and orotic acid, in pharmaceutics in order to improve physico-chemical properites such as solubility in water and to enhance the stability of the drug. But the physico-chemical properties of the salt drug with racemic lysine are much different from that of salt drug with L-lysine. While L-lysine salt of ibuprofen is well crystallized, L-lysine salt of acetylsalicylic acid shows poor crystallinity. In the case of aspirin, racemic lysine is preferable in forming its salt since the D, L-lysine salt of aspirin is easily dried and well crystallized after reaction using small amounts of water and ethanol solvent (Yamada *et al.*, 1981). Various methods for racemization of lysine have been reported (Rhee *et al.*, 1994a, b). For example, the optically active amino acid can be racemized i) by heating with water in the presence of strong base or strong acid, ii) by mild heating (80-100°C) with acetic acid in the presence of catalytic amounts of salicyaldehyde (Yamada *et al.*, 1983), iii) by heating lysine with phosphoric acid.

Enantiomeric resolution is an important field in analytical chemistry, especially in pharmaceutical analysis. Most of the synthetic chiral drugs are sold in the form of racemates. New single-isomer products on the chiral drug market require enantiomeric intermediates and enantioselective technologies (Stinson 1994). For the investigation of the metabolism of chiral drug racemate, HPLC (Oi *et al.*, 1984; Kuropka *et al.*, 1989) and CE (Heuermann *et al.*, 1993) are widely used. Enantiomeric separation can be achieved by using either direct (Frank *et al.*, 1978) or indirect methods. One of the direct methods is using a chiral stationary phase, while the indirect methods (Liu *et al.*, 1981; Liu *et al.*, 1982) are based on chiral derivatization, i.e. conversion of the enantiomers into diastereomers, and separations using achiral stationary phase. Enantiomers of amino acid mixtures can be resolved directly in a fairly short time by GC (Frank *et al.*, 1980; Makita *et al.*, 1982) using a capillary themal column coated with chirasil-val(Frank *et al.*, 1978) and an optically active stationary phase with high ther-

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mal stability. Amino acids are derivatized into their N(O,S)-trifluoroacetyl isopropyl ester or N(O,S)-pentafluoropropionyl isopropyl ester for resolution into the D and L enantiomers (Bailey *et al.*, 1980; Abe *et al.*, 1981; Liardon *et al.*, 1980; Abe *et al.*, 1983). We investigated a simple, practical and economical process for racemization of lysine and the optimum conditions for derivatization and separation of the lysine enantiomers by GC-Mass spectroscopy using a chirasil-val column.

MATERIALS AND METHODS

Reagents

D and L-lysine were purchased from Sigma Chem. (USA). L-leucine as internal standard and pentafluoropropionic acid (PFPA) were purchased from Alltech associate inc. (USA). Salicylaldehyde, 85%-phosphoric acid and trifluoroacetic anhyride (TFA) were obtained from Aldrich Chem. (USA). 5N-HCl in 2-propanol (isopropyl alcohol; IPA) was obtained from Janssen Chimica. (Belgium). Chromatographically pure methylene chloride and ethyl acetate were bought from Wako Pure Chem. (Japan) and were re-distilled in all-glass apparatus before use. Acetic acid, first grade reagent, was purchased from Duk San Pharma. (Korea).

Apparatus

A chirasil-val capillary column (25 m × 0.25 mm i.d., Alltech, USA) was installed in a GC-3400 (Varian, USA) equipped with mass spectrometer and FID.

Table I. Analytical conditions of GC-MS for chiral separation

GC	MS
Model: Varian GC-3400 with FID	Model: Finnigan Mat TSQ 700 Quadrupole type
Column: 0.25 mm id × 25 m (Alltech)	Used method
Temperature	Q1 single stage MS
Injector: 250°C	Ionization
Detector: 250°C	EI method
Oven program	Energy: 70 eV
Initial temp.: 100°C	Scan
(hold time 1 min)	Range: LMR 0-400 amu
Rate: 3°C /min	Rate: 0.56/sec
Final temp.: 200°C	Temperature
(hold time 7.06 min)	Interface temp.: 280°C
Gas	Ion source temp.: 150°C
Carrier: N_2 4 ml/min	Dynode: -15.0 KV
Make up: N_2 40 ml/min	
Combustion: H_2 30 ml/ Air 300 ml/min	

Spectra-Physics 4270 was used for data processing. Mass spectrometry was performed on a TSQ 700 (Finnigan Mat, USA) using a GC-EI method. The instrumental conditions are tabulated in Table I.

Derivatization of racemic lysine for GC-MS analysis

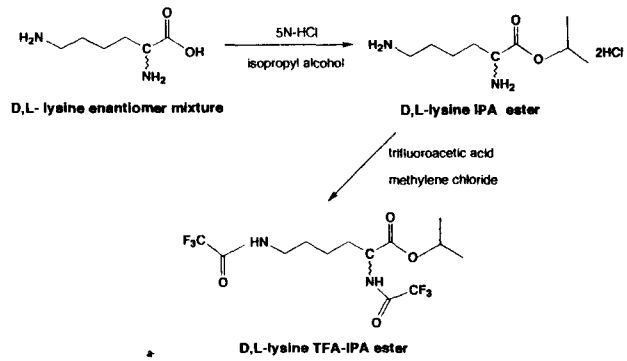
A racemic lysine (4 mg) and L-leucine (4 mg) as internal standard were placed in cap vial. 4 ml of 5N-HCl in anhydrous isopropanol was added and then tightly sealed with a teflon faced liner. The reaction mixture was heated at 110°C for 3 hr. After the reaction, excess reagent was removed under a gentle stream of nitrogen gas with cooling. 0.7 ml of TFA (or PFPA) and 1 ml of methylene chloride were added to the sample in a heavy-walled cap vial. The mixture was heated to 110°C for 15 min. Excess reagent and solvent were removed under a gentle stream of nitrogen gas at 40°C. The dry residue was dissolved in an appropriate volume of ethyl acetate and 1 μl aliquots were injected into the GC-FID or MS Detector. Above derivatization process of analyte is shown in Scheme 1.

RESULTS AND DISCUSSION

Chiral separation of racemic lysine

There are growing concerns for enantioselective technology to meet the demand for enantiomerically active compounds. D and L-lysine with the same chemical and physical properties except for the direction of plane-polarized light rotation are illustrated in Fig. 1.

The general structure of chirasil-val stationary phase is given in Fig. 2 (Farnk *et al.*, 1978). Chirasil-val is a silicone-based polymer with chiral functional groups incorporated into the "backbone". The two enantiomers to be separated interact with the chiral stationary phase via hydrogen bonds to form diastereomeric association complexes with differences of sev-



Scheme 1. Derivatizatim of racemic lysine for gas chromatography.

eral hundred calories in their solvation enthalpy.

In order to test the linearity of the method, 0-2.5 mg of L (or D)-lysine was derivatized and analyzed by GC. Calibration graphs for L or D-lysine were obtained by plotting the ratios of their peak area of the internal standard to that of D or L-lysine and they were linear in the range of 0-2.5 mg. The lines obtained by the least square method (Jung *et al.*, 1994) had values of r^2=0.999 for D-lysine and r^2=0.993 for L-lysine indicating reliable correlation.

Optimum conditions for derivatization

The derivatization methods of the enantiomers with PFPA and TFA were compared to each other as shown in Table II. Retention time of D-lysine was slightly faster than that of L-lysine in both methods. The retention time was decreased by replacing TFA with PFPA and the enantiomeric resolution factor (Abe *et al.*, 1981) was increased by replacing PFPA with TFA. This result is likely to be the consequence of steric hindrance such as reduction of stereospecificity between the perfluoroacyl group and chiral stationary phase. The TFA derivative with good reproducibility and low volatility was found to be more effective than the PFPA derivative.

Reproducibility of lysine derivatization is essential to obain a reliable result. Therefore, a constant amount of D or L-lysine standard samples to be analyzed by GC was derivatized 3 times. The reproducibility was satisfied with C.V.=2.66% for L-lysine and 6.16% for D-lysine as shown in Table III. Fig. 3 shows the chromatogram of D, L-lysine TFA-IPA ester by using GC-MS spectrometer. D and L-lysine were cleary separated.

The quantiation of D and L-lysine by GC proved to be very sensitive to be small changes in the analytical procedure, but it requires a control of rigid and time-consuming experimental conditions for a routine method. To establish the optimum conditions of lysine derivatization for chiral separation, quantitations of D and L-lysine were performed with various reaction conditions. The extent of derivatization of lysine was not affected by small amounts (1-7 ml) of esterificational reagent, 5N HCl in anhydrous isopropanol, but it increased with reaction time for esterification. We found that the D and L-lysine remained in the solid state after the reaction for 1 hr and completely dissolved in IPA for 3 hr. In recent papers, the reaction time for esterification of lysine is not more than 1 hr (Farnk et al., 1978; Bailey *et al.*, 1980; Abe *et al.*, 1981; Liardon *et al.*, 1980), but the above result indicated that at least 3 hr is required to be completely derivatized. The derivatization of lysine seems to be dependent on the slow first step which is the este-

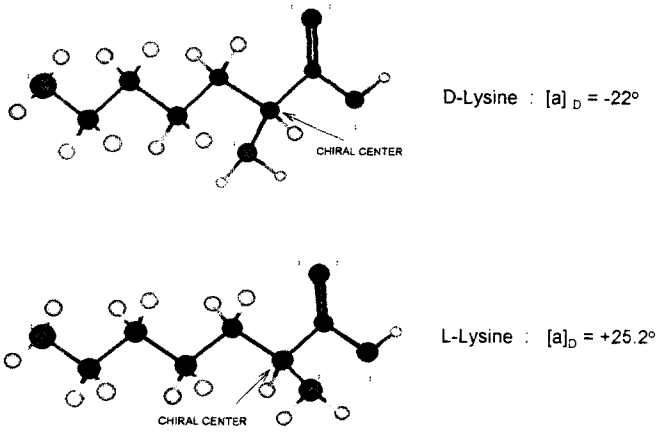


Fig. 1. Molecular Structure of D-, L-Lysine.

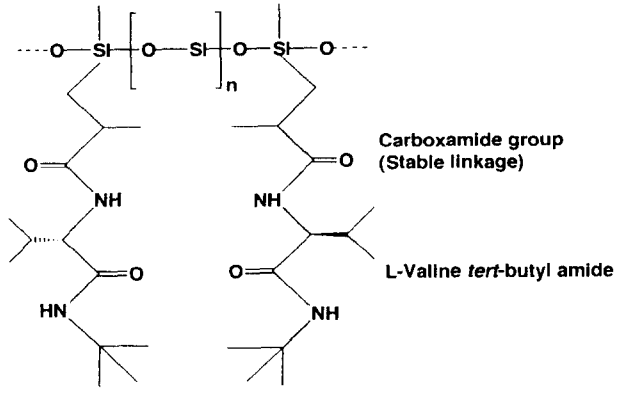


Fig. 2. Structure of chiral stationary phase of Chirasil-Val. L-valine tert.-butyl-amide is linked to the polysiloxane via the highly stable carboxamide group. *n: Should be more than 7.

Table II. Retention times and resolution factors TFA and PFPA derivatives

Reagent used for acylation	Retention time (min)		Resolution factor[a]
	D-lysine	L-lysine	
TFA	29.53	30.04	5.10
PFPA	26.29	26.72	4.78

[a]Resolution factor: $R=2(t_L-t_D)/(W_L-W_D)$
t_L, t_D: retention times of L and D-lysine
W_L, W_D: peak widths of L and D-lysine.

Table III. Reproducibility of D, L-lysine derivatization

Analytical No.	Degrees of derivatization[a]	
	D-lysine	L-lysine
1	0.372	0.458
2	0.321	0.449
3	0.357	0.478
Mean±SD	0.350±0.021	0.462±0.012
Coefficient of Variance (%)	6.16	2.66

[a]Lysine peak area/Leucine peak area (Internal standard).

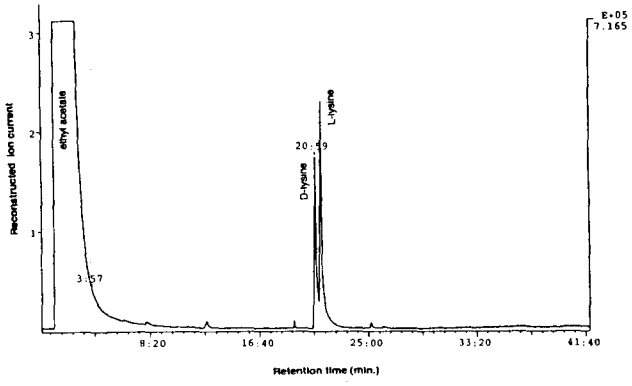


Fig. 3. Gas chromatogram of D, L-lysine TFA-IPA ester separated by Chirasil-Val column. Instrument: Varian 3400 GC-TSQ 700 MS. Analytical conditions are shown in Table 1.

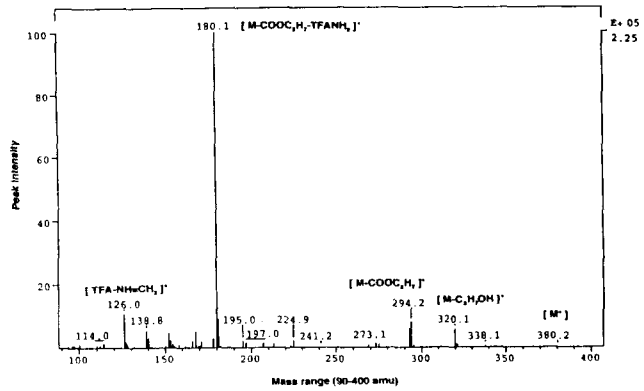


Fig. 4. Mass spectrum of lysine TFA-IPA ester.

Table IV. Degrees derivatization of lysine in various reaction conditions

Reactional conditions	n[a]	Degrees of derivatization[b]	
		D-lysine	L-lysine
Amount of 5N-HCl in IPA			
1 ml	3	0.429±0.019	0.339±0.050
4 ml	3	0.350±0.021	0.460±0.012
7 ml	3	0.179±0.076	0.351±0.008
Time of esterification			
1 hr	3	0.350±0.021	0.460±0.012
3 hr	3	0.610±0.053	0.597±0.039
Temp. & time of acylation			
110°C for 15 min	3	0.350±0.021	0.460±0.012
room temp. for 1 hr	3	0.332±0.095	0.338±0.056

[a]Number of analysis
[b]Lysine peak area/Leucine peak area (Internal standard)

rification with IPA, while the second step which is the acylation with TFA or PFPA was very fast. Also, two conditions of temperature and time for acylation were used i) by heating to 110°C for 15 min (Liardon *et al.*, 1980), ii) by reacting at room temperature for 30-60 min (Rhee *et al.*, 1994). The former showed a higher degree of derivatization of lysine than the latter. The results are tabulated in Table IV.

Mass spectral data of TFA-IPA ester of lysine

To confirm the fragmentation pattern of the enantiomers, GC-Mass spectroscopy using single quadrupole scan-EI method was performed. Fig. 4 shows the EI spectrum of lysine TFA-IPA ester at 70 eV. The M^+ ion peak of lysine TFA-IPA ester [*m/z* 380.2] was hardly observed, while the base peak of M-$COOC_3H_7$-$TFANH_2$ [*m/z* 180.1] was found with strong intensity. The *m/z* 320 peak represents a C_3H_7OH elimination and the *m/z* 294 peak represents $COOC_3H_7$ elimination from the molecular ion. The *m/z* 126 peak represents the $TFA^+NH{=}CH_2$ ion. The other peaks smaller than *m/z* 126 are produced by secondary bond cleavages.

Comparison of racemizational methods

Two racemization methods were studied in this experiment. Possible mechanisms for racemization of α-amino acids (Matsuo *et al.*, 1970) are initial protonation of its imine (Yamada *et al.*, 1983) or amine, followed by proton abstraction from the α-carbon atom by acetate anion or phosphoric anion. The carbanion (Matsuo *et al.*, 1970) formed by proton abstraction from the α-carbon atom act as a nucleophile and racemization occurs from the electrophilic attack (H^+) to either side of the plane of the carbanion.

One method using phosphoric acid is as follows; L-lysine (6.2 g) was dissolved in water (15 ml) and 85% phosphoric acid (5 ml), and then heated to reflux at 164°C. After the reaction, lysine was diluted and loaded on a column of cation-exchange resin. 5% ammonia elutriate was evaporated to dryness. Racemic lysine was precipitated in hot 95% ethyl alcohol to give 5.2 g of product (83.9% yield). To follow the changes from L-lysine to racemic lysine, samples were taken at various intervals. Fig. 5 shows the racemization ratio by the method using phosphoric acid. In general, this racemization process was reported to be performed within the range of 110-210°C (Emmick *et al.*, 1952) and it was carried out at 160°C in this study.

The other method using acetic acid is as follows; L-lysine (6.0 g) was dissolved in acetic acid (180 ml) containing 0.05 molar equivalent of salicylaldehyde (0.22 ml), and heated at 100°C with stirring. After 1 hr, the reaction mixture was treated with active charcoal and concentrated to give 5.9 g of product (98% yield). The changes from L-lysine to racemic lysine by this method are shown in Fig. 6. In this case, racemization of lysine is complete within 50 min and found to be accelerated in acetic acid in the presence of a catalytic amount of salicylaldehyde.

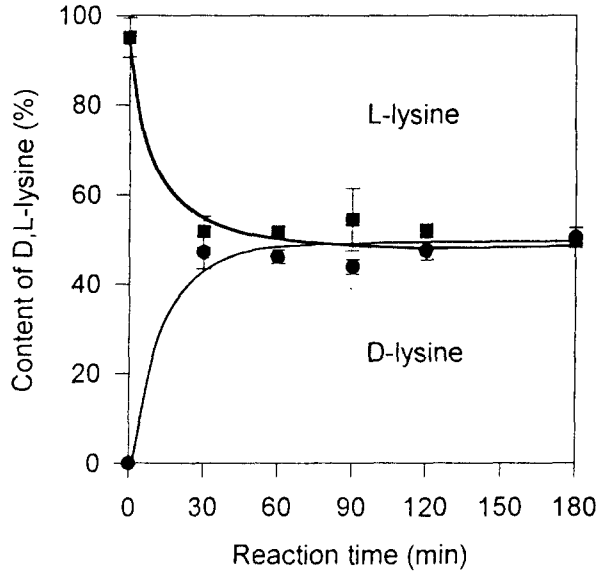


Fig. 5. Racemization ratios of lysine by phosphoric acid treatment. Each spots is given mean±SD by 5 determinations.

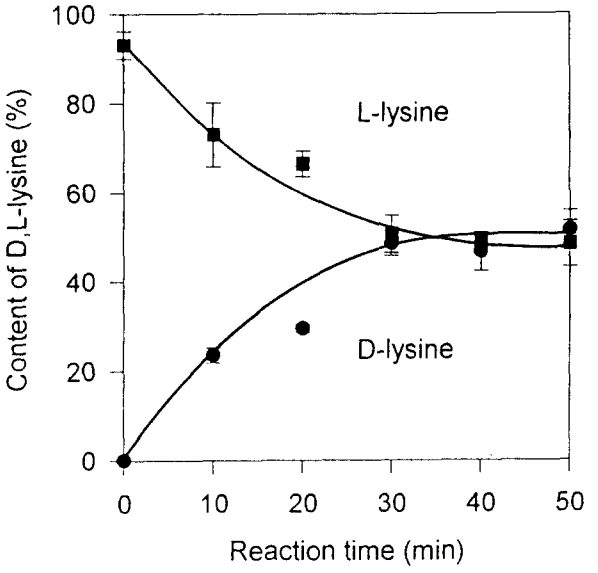


Fig. 6. Racemization ratios of lysine by acetic acid treatment. Each spots given as mean±SD by 5 determinations.

Although racemization using acetic acid occurred under mild conditions (80-100°C) and gave some advantages such as high yield and purity, salicylaldehyde was required for the process as catalyst. The method using phosphoric acid is preferable to that of using acetic acid, due to some advantages such as the rapid process for the racemization, recovering the racemic lysine from the reaction medium, reutilization of phosphoric acid without further treatment, and no need for a catalyst. Both methods are finished within 1 hr completely.

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