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N-Ethoxycarbonylation combined with (*S*)-1-phenylethylamidation for enantioseparation of amino acids by achiral gas chromatography and gas chromatography–mass spectrometry

Man-Jeong Paik[a,b], Jinwoo Lee[c], Kyoung-Rae Kim[c,*]

[a] *Metabolomic Analysis Laboratory, Institute for Neuroregeneration and Stem Cell Research, School of Medicine, Ajou University, Suwon 443-721, South Korea*
[b] *Department of Molecular Science and Technology, School of Medicine, Ajou University, Suwon 443-721, South Korea*
[c] *Biometabolite Analysis Laboratory, College of Pharmacy, Sungkyunkwan University, Suwon 440-746, South Korea*

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ABSTRACT

N-Ethoxycarbonylation was combined with (*S*)-1-phenylethylamidation for enantioseparation of amino acids by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) on achiral capillary columns. The method provided complete enantioseparations of 12 amino acids as diastereomeric *N*-ethoxycarbonyl/(*S*)-1-phenylethylamides with exceptional resolutions for proline ($R_s \geq 9.9$) and pipecolic acid ($R_s \geq 10.2$). GC–MS analysis in selected ion monitoring mode employing standard addition method, facilitated quantitation of D-pipecolic acid in kidney bean (0.95 μg/10 mg) and adzuki bean (0.14 μg/10 mg). The peak area ratios indicated that they had the identical chiral composition at 2.5% for D-pipecolic acid and 97.5% for L-pipecolic acid.

1. Introduction

Over the last decade, with the increasing discovery of D-amino acids in diverse samples [1–10], it has become utmost important to know whether peptides or proteins contain D-amino acids and to find their presence in free forms as well. The D-amino acids in these peptides are essential for their biological activities because the change of D-amino acid to L-amino acid would lose their biological activities. The accurate determination of their absolute configuration has become an important task for the study on their biochemical roles [1–3,8,11,12].

Accurate chiral discriminations of amino acids require the use of high-resolution GC, HPLC or CE under direct chiral conditions or indirect achiral conditions as well documented in the recent review articles [13–18]. When employing GC combined with MS to achieve higher enantiomeric resolution, both direct and indirect approaches require amino acids to be preconverted into volatile derivatives [3,4–8,10,19,20]. In consideration of this prerequisite, indirect enantioseparation as volatile diastereomeric derivatives using conventional achiral stationary phases with excellent thermal stability and thus long-term durability appears to be preferred over the direct approach [5,20]. Most of tailor-made chiral capillary columns are known to be thermally unstable, thereby requiring to convert amino acids into much more volatile derivatives [3,4,6–10,20].

In recent years, zwitterionic amino acids have been efficiently recovered from aqueous solutions into organic solvents employing simultaneous alkoxycarbonyl (AOC) reaction and esterification for their enantioseparations in direct approach [3,10,20] and in indirect approach [21]. However, this rapid one-step method still has limitation to be directly applied to the aqueous amino acid extracts without prior reduction to submilliliters. This problem was previously overcome by employing solvent extractive two-phase ethoxycarbonyl (EOC) reaction with ethyl chloroformate (ECF), followed by diastereomeric esterification with chiral (*S*)-(+)-3-methyl-2-butanol in the enantioseparation of 30 racemic amino acids in a single analysis [5]. All the racemic amino acids were resolved into their enantiomeric pairs with high resolutions ($R_s \geq 1.8$) except for proline ($R_s = 1.5$) and pipecolic acid ($R_s = 1.7$). The esterification of carboxyl groups was, however, very

* Corresponding author. Tel.: +82 31 2907703; fax: +82 31 2907723.
E-mail address: krkim@skku.edu (K.-R. Kim).

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slow, thus requiring extended heating (100 °C for 1.5 h) compared with the instantaneous *N*-EOC reaction. In a previous report [21], as a different approach, the amidation of carboxyl groups in chiral profens with chiral (*R*)-1-phenylethylamine (PEA) under ECF/triethylamine activation was preferentially employed because more stable diastereomeric amides were formed at room temperature within a few minutes. Moreover, the rigidity of amide bond attached to bulky 1-phenylethyl group provided high enantiomeric resolutions (R_s = 2.5 – 5.6). Hence, *N*-EOC reaction combined with diastereomeric 1-phenylethylamidation appears to be more suitable for rapid chiral discrimination of amino acids. However, this attempt has been rarely made to date.

As a new attempt in this study, diastereomeric amidation with (*S*)-1-PEA following the previous *N*-EOC reaction [5] was investigated for the enantioseparation of amino acids. Each amino acid as *N*-EOC/(*S*)-1-phenylethylamide was subjected to GC analyses on the achiral DB-5 and DB-17 dual-columns of different polarity. And each chemical structure was confirmed by GC–MS. The present method was intended to complement the previous diastereomeric esterification method [5] for the complete enantiomeric resolution of proline and pipecolic acid. In this study, chiral composition tests were performed on kidney bean and adzuki bean for pipecolic acid by GC–MS in selected ion monitoring (SIM) mode employing the method of standard addition.

2. Experimental

2.1. Materials

The following 12 racemic amino acid standards and 11 enantiomerically pure standards were obtained from various vendors such as Sigma–Aldrich (St. Louis, MO, USA): alanine, valine, leucine, isoleucine, proline, α-aminobutyric acid, β-aminoisobutyric acid, pipecolic acid, norleucine, norvaline, 2,3-diaminopropionic acid, *N*-methylaspartic acid, L-alanine, L-valine, L-leucine, L-isoleucine, L-proline, L-α-aminobutyric acid, L-norvaline, L-norleucine, L-2,3-diaminopropionic acid, L-pipecolic acid and *N*-methyl-L-aspartic acid. (*S*)-1-PEA (99.5%) used as the chiral reagent was purchased from Sigma–Aldrich, triethylamine (TEA) and ECF from Janssen (Geel, Belgium), and *n*-hydrocarbon standards (C_{18}–C_{40}, even numbers only) from Polyscience (Niles, IL, USA). Diethyl ether, acetonitrile, ethyl acetate, toluene, dichloromethane and isooctane of PCB grade were purchased from Kanto (Tokyo, Japan), sodium hydroxide from Kokusan (Tokyo, Japan), sulfuric acid from Songyawon (Osaka, Japan) and hydrochloric acid from Samchun (Pyeongtaek, South Korea). All other chemicals were of analytical-reagent grade and used as received. Kidney bean and adzuki bean were locally purchased and desiccated at −20 °C until being used for the chiral composition tests of pipecolic acid and for the quantitation of D-enantiomer.

2.2. Solutions of amino acids, hydrocarbons and other reagents

Each standard stock solution of amino acids was made up at 10 μg/μl in 0.1 M HCl. The working solutions at varied concentrations were then prepared by diluting each stock solution with 0.1 M HCl. Calibration samples were prepared at the amount ranges from 0.25 to 2.5 μg by mixing appropriate aliquots of each working solution. A mixed hydrocarbon solution containing 12 *n*-hydrocarbons (C_{18}–C_{40}, even numbers only), each at 1.0 μg/μl in isooctane, was used as the I.S. solution for retention index (*I*) measurement. For method validation works, a hydrocarbon I.S. solution was made to contain two *n*-hydrocarbons (C_{18} and C_{26}) each at 0.1 μg/μl in isooctane. L-Norvaline solution used as I.S. solution for pipecolic acid quantitation was prepared at 0.1 μg/μl in 0.1 M HCl. (*S*)-1-PEA solution was prepared at 0.5 M in methanol. TEA and ECF solutions were prepared in acetonitrile at 50.0 and 60.0 mM, respectively. All standard solutions prepared were stored at 4 °C except for the amino acid stock solutions which were stored in frozen condition.

2.3. Gas chromatography and gas chromatography–mass spectrometry

The GC analyses were performed with an Agilent 6890 gas chromatograph, equipped with electronic pneumatic control system, a split/splitless inlet system, an automatic sampler, two flame ionization detection systems and GC Chemstation (Agilent Technologies, Atlanta, GA, USA). The oven was installed with two columns made of DB-5 (SE-54 bonded) and DB-17 (OV-17 bonded) fused-silica capillary columns (30 m × 0.25 mm I.D., 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA). The injector and detector temperatures were 260 and 300 °C, respectively. Samples (ca. 1.0 μl) were injected in the splitless mode with purge delay time of 42 s. The flow rate of helium as carrier gas was set at 1.0 ml/min in constant flow mode. The oven was initially at 80 °C (2 min), programmed at 20 °C/min to 150 °C, then to 230 °C at 3 °C/min and finally at 10 °C/min to 290 °C (20 min). A standard solution of *n*-hydrocarbons (C_{18}–C_{40}, even numbers only) in isooctane was co-injected with samples to compute temperature-programmed *I* values according to the following equation: $I = 100z + 100(y - z)\ \{[t_{R(x)} - t_{R(z)}]/[t_{R(y)} - t_{R(z)}]\}$, where $t_{R(x)}$, $t_{R(z)}$, and $t_{R(y)}$ were the retention times of analyte x, the hydrocarbon with *z* carbon number eluting before x and the hydrocarbon with *y* carbon number eluting after x, respectively, while z and y were the carbon numbers of the hydrocarbon with $t_{R(z)}$ and $t_{R(y)}$, respectively. All GC runs were performed in triplicate.

GC–MS analyses in both scan and SIM modes were performed using an Agilent 6890 gas chromatograph, interfaced with an Agilent 5973 mass-selective detector (70 eV, electron impact mode) and installed with an Ultra-2 (5% phenyl–95% methylpolysiloxane bonded phase; 25 m × 0.20 mm I.D., 0.11 μm film thickness) cross-linked capillary column (Agilent Technologies, Atlanta, GA, USA). The temperatures of injector, interface and ion source were 260, 300 and 230 °C, respectively. Helium was used as carrier gas at a flow rate of 0.5 ml/min with constant flow mode. Samples were introduced in the split-injection mode (10:1) and the oven temperature was set initially at 150 °C (2 min), then programmed at 10 °C/min to 180 °C, then to 230 °C at 3 °C/min and finally to 300 °C (20 min) at 10 °C/min. The mass range scanned was 50–650 U at a rate of 0.99 scan/s. In the SIM mode, the base peak ions at *m*/*z* 156 and at *m*/*z* 144 were used for the quantitation of D-pipecolic acid and L-norvaline (used as I.S.), respectively. A dwell time of 100 ms was chosen and the relative voltage of electron multiplier was set to 200 V higher than that in the scanning mode (1000 V). And the oven temperature was maintained at 80 °C (2 min), then programmed at 30 °C/min to 240 °C, then to 270 °C at 5 °C/min and finally to 300 °C (2 min) at 10 °C/min. All the GC–SIM–MS runs were performed in triplicate.

2.4. Sequential N-ethoxycarbonylation/(S)-1-phenylethylamidation

An aliquot (1 ml) of distilled water containing amino acids in racemic forms at varied amounts was added to dichloromethane (1 ml) spiked with neat ECF (20 μl) and I.S. solution (*n*-C_{18} and *n*-C_{26}, each at 0.5 μg). The aqueous phase was alkalinized (pH ≥ 10) with 5 M NaOH and the mixture was immediately vortex-mixed (10 min) at room temperature to conduct the first-step reaction in the three-step reaction scheme (Fig. 1). The upper aqueous layer was acidified (pH ≤ 2) with conc. sulfuric acid and saturated