Original Article

# การแยกอิแนนทิโอเมอร์ของฟีนิลไกลซีนโดยโครมาโทกราฟีแบบแลกเปลี่ยนลิแกนด์

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## บทคัดย่อ

**บทนำ:** เนื่องจากฟีนิลไกลซีนในโครงแบบ “ดี” เป็นสารที่มีความสำคัญทางเภสัชกรรมโดยใช้ในการสังเคราะห์แอมพิซิลลินและเซฟาเลกซิน ดังนั้น จึงจำเป็นต้องประเมินความบริสุทธิ์ทางอิแนนทิโอเมอร์ก่อนนำไปใช้ การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาวิธีการแยกไครัลสำหรับฟีนิลไกลซีนแทนการใช้คอลัมน์โครมาโทกราฟีของเหลวสมรรถนะสูงชนิดไครัลที่มีราคาแพง โดยการใช้วัฏภาคเคลื่อนที่ที่ประกอบด้วยคอปเปอร์และแอล-โพรลีนร่วมกับวัฏภาคคงที่ ได้แก่ คอลัมน์ชนิด C18 **วิธีดำเนินการวิจัย:** ทำการศึกษาหาสภาวะที่เหมาะสมสำหรับการแยก ดี- และแอล-ฟีนิลไกลซีน ได้แก่ ค่าพีเอชของวัฏภาคเคลื่อนที่ ความเข้มข้นของคอปเปอร์และแอล-โพรลีนและร้อยละของเมทานอลในวัฏภาคเคลื่อนที่ และประเมินประสิทธิภาพของการแยกจากค่าการแยก ระหว่างพีกของไอโซเมอร์ดีและแอล ตลอดจนลักษณะของโครมาโทแกรม รวมทั้งสร้างกราฟมาตรฐานเพื่อหาช่วงและความเป็นเส้นตรง **ผลการวิจัย:** เมื่อใช้สารผสมซึ่งประกอบด้วยคอปเปอร์และแอล-โพรลีนความเข้มข้น 2.5 และ 5 มิลลิโมลาร์ ในเมทานอลร้อยละ 10 ที่มีค่าพีเอชเท่ากับ 6 เป็นวัฏภาคเคลื่อนที่ พบว่า ดี- และแอล-ฟีนิลไกลซีนแยกจากกันได้ดีโดยปรากฏพีกที่เวลาประมาณ 5 และ 11 นาที ตามลำดับ ภายใต้สภาวะที่เหมาะสมนี้ ความเข้มข้นกับพื้นที่ใต้พีกมีความสัมพันธ์เชิงเส้นกันดี โดยมีค่าสัมประสิทธิ์สหสัมพันธ์เท่ากับ 0.9997 และ 0.9998 สำหรับ ดี-ฟีนิลไกลซีนและแอล-ฟีนิลไกลซีน ตามลำดับ **สรุปผลการวิจัย:** วัฏภาคเคลื่อนที่ที่ประกอบด้วยคอปเปอร์ แอล-โพรลีน เมทานอล และมีค่าพีเอชที่เหมาะสม ร่วมกับคอลัมน์ชนิด C18 สามารถใช้ในการแยกอิแนนทิโอเมอร์ของฟีนิลไกลซีน ดังนั้นจึงสามารถนำสภาวะนี้ไปพัฒนาและตรวจสอบความถูกต้องของวิธีวิเคราะห์หาปริมาณ ดี- และแอล-ฟีนิลไกลซีนที่ทำได้ง่ายและมีราคาถูกในลำดับต่อไป

**คำสำคัญ:** การแยกอิแนนทิโอเมอร์, ฟีนิลไกลซีน, โครมาโทกราฟี, การแลกเปลี่ยนลิแกนด์

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# Enantioseparation of Phenylglycine by Ligand Exchange Chromatography

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## Abstract

**Introduction:** Since phenylglycine in "D" configuration is pharmaceutically used for the synthesis of ampicillin and cephalexin, its enantiomeric purity needs to be confirmed prior to use. This study aimed to develop a chiral separation method for phenylglycine instead of using expensive chiral chromatographic columns. It was based on the use of a chiral mobile phase consisting of copper(II) and L-proline with a typical C18 column as a stationary phase. **Methods:** The optimal conditions for the separation of D- and L-phenylglycine, i.e. the pH of the mobile phase, concentration of copper(II) and L-proline, and percentage of methanol as an organic modifier in the mobile phase, were studied. The performance of the separation was evaluated by the resolution between the peaks of D- and L-isomers as well as the characteristics of the chromatograms. A standard curve was created to investigate the range and linearity. **Results:** D- and L-phenylglycine best separated from each other with the retention time of about 5 and 11 min, respectively, when a mixture containing 2.5:5 mM copper (II):L-proline, 10% methanol, and with a pH of 6.0 was used as the mobile phase. Under these optimal conditions, satisfactory linear relationships between the concentration and peak area were obtained for both D- and L-phenylglycine with correlation coefficients of 0.9997 and 0.9998 respectively. **Conclusion:** A copper(II)/L-proline mixture at a suitable concentration and pH in the presence of an appropriate amount of methanol could be effectively used as a mobile phase together with a C18 column for the separation of phenylglycine enantiomers. Thus, these optimal conditions can be used for the further development and validation of a simple and inexpensive method for the quantitation of D- and L-phenylglycine.

**Keywords:** Enantioseparation, Phenylglycine, Chromatography, Ligand-exchange
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## Introduction

Chirality is a major concern in modern pharmaceutical industry (Lam et al., 1992) because enantiomers of drugs may have different pharmacological activities, toxicity as well as pharmacokinetic and pharmacodynamic effects (Lam et al., 1992). By this reason, the separation of enantiomeric compounds has become increasingly important in both analytical and preparative aspect (Hu et al., 2013, Wang et al., 2012 and Wang et al., 2011). Among the analytical separation techniques, high performance liquid chromatography (HPLC) is the most convenient, reproducible and widely applicable method for the separation of chiral molecules such as drugs, amino acids and sugars. (Davankov et al., 1983, Pirkle et al., 1989 and Husain et al., 1994). The chiral resolution mechanism is based on the transient formation of a pair of diastereomers which have different chemical properties and consequently different retentivities. For this purpose, enantiomers are subjected to chromatography either on a chiral stationary phase (CSP) or in chiral mobile phase (CMP) i.e. chiral-ligand exchange chromatography (CLEC). From last two decades, CLEC has been applied to the analysis of various compounds (Mericko et al., 2007 and Marchelli et al., 2007) by incorporating L-proline as a chiral selector in the mobile phase (Yamazaki et al., 1998, Sotgia S et al., 2008 and Wu et al., 2014). This compounds combined with copper(II) salts i.e. copper(II) sulfate and mixtures of D,L-enantiomers to form diastereoisomeric ternary complexes with the different retention ability the column of reversed phase liquid chromatography.

Phenylglycine (PHG) is an amino acid which has one chiral carbon in its structure (Figure. 1). D-phenylglycine (D-PHG) has a valuable property for use as a substrate for the synthesis of ampicillin and cephalexin (Victery et al., 1998) while L-phenylglycine (L-PHG) is used as a chiral selector in chiral column (Pirkle® column). Nowadays, the enantiomers of PHG could be separated by CSP i.e. Chirex 316 column (Sajewicz et al., 2014), cellulose tris(3,5-chlorophenylcarbamate) (Jin et al., 2008) or crown ether column (Zokowski et al., 1991), however, the high price of these types of columns limits their practical use for many laboratories.

From the literature review, copper(II) reacts with L-proline at the molar ratio of 1:2 (Nazareth et al., 2002) to form the binary complex. When the enantiomeric mixtures are submitted to the analytical process, one molecule of L-proline can be replaced by one molecule of D-PHG or L-PHG to form the ternary complex composed of copper (II), L-proline and D-PHG or L-PHG. The chromatographic separation occurs as the result of the different properties of two ternary complexes i.e. complex stability and retention ability. Therefore, the purpose of this study was to investigate the facile and inexpensive method for the separation of enantiomeric mixtures of PHG. Various parameters such as the concentration of the copper(II) and L-proline, pH of mobile phase and the concentration of organic modifier in mobile phase i.e. methanol were optimized to achieve the

optimal conditions, giving the satisfactory separation performance as well as economical feature.

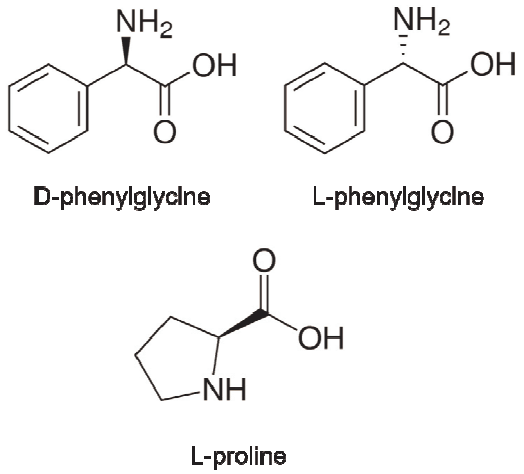


**Figure. 1** Chemical structures of D-PHG, L-PHG and L-proline.

## Materials and Methods

### Materials

L-PHG (as white crystal powders), D-PHG (as white powders), L-proline (as white powder) and copper(II) sulfate (analytical reagent grade) were purchased from Sigma (St. Louis, Missouri, USA). Methanol (HPLC grade) and acetonitrile (HPLC grade) were obtained from Honeywell® (Morris Plains, New Jersey, USA). All other chemicals were of analytical grade from Merck (Darmstadt, Germany). Deionized water was used to prepare all solutions.

### HPLC Instrumentation

The HPLC assays for the content of D-PHG and L-PHG were carried out by using the system consisting of an Agilent 1100 Series equipped with a diode array detector and Reprosil-Pur® Basic C18 column (150 mm × 4.6 mm, I.D.; 5 µm particle size). The flow rate was 1 mL/min. The column temperature was maintained at 25 °C. UV detection was set at 210 nm and the injection volume was 20 µl.

### Preparation of standard solution

To prepare the standard stock solutions, D-PHG and L-PHG were prepared as a mixture solution containing 5 mM D-PHG and 5 mM L-PHG by dissolving 0.0075 g D-PHG and 0.0075 g L-PHG in 10 mL of deionized water.

### Optimization of the analytical procedure

The experimental parameters which affected the enantioseparation were studied. These parameters included the concentration of the copper(II) and L-proline (1:2, 2.5:5 and 5:10 mM), pH of the mobile phase (4, 5, 6, 7 and 8) and the concentration of methanol in the mobile phase (5, 10, 15 and 20% v/v). A standard solution containing 0.5 mM D-PHG and 0.5 mM L-PHG was used as a sample for the injection in this study. The performance of chromatogram was evaluated by resolution and retention time (RT).

### Study of linearity and range

After the optimal conditions were established, standard curves of D-PHG and L-PHG were constructed. Different amounts (20, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µL) of 5 mM stock standard solution were accurately transferred into a series of 1.5 mL microcentrifuge tubes by the use of micropipettes.

Water in different volumes was precisely added into each tube to bring the total volume to 1 mL. This resulted in a set of standard D-PHG and L-PHG solutions with the concentration of 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25 and 2.50 mM. All samples were filtered with 0.45 µm membrane filter prior analysis by HPLC.

## Results

### Effect of the concentration of the copper(II) and L-proline at a specific copper(II)-L-proline ratio

To investigate the effect of copper(II) and L-proline concentrations, the concentrations were varied by fixing at the constant ratio (1:2, 2.5:5 and 5:10 mM) The pH or of the mobile phase and percent of organic modifier were fixed at 6 and 5% methanol, respectively. As shown in Figure. 2(c), the separation of enantiomeric mixtures could not appear when the concentration of copper(II) and L-proline was 5:10 mM. Besides, the background color of mobile phase at this copper(II) to L-proline ratio was very intense, thus interfering the sample signals and lowering the sensitivity. The use of 2.5 mM copper(II) and 5 mM L-proline gave a good characteristic of chromatogram with satisfactory resolution (Figure. 2(b)). Although, the enantiomeric mixtures could be separated at the ratio of 1:2 mM, the peak of L-proline was close to the unknown negative peak at about 15 min (Figure. 2(a)). So, the copper(II) to L-proline ratio at 2.5:5 mM was used for subsequent studies.

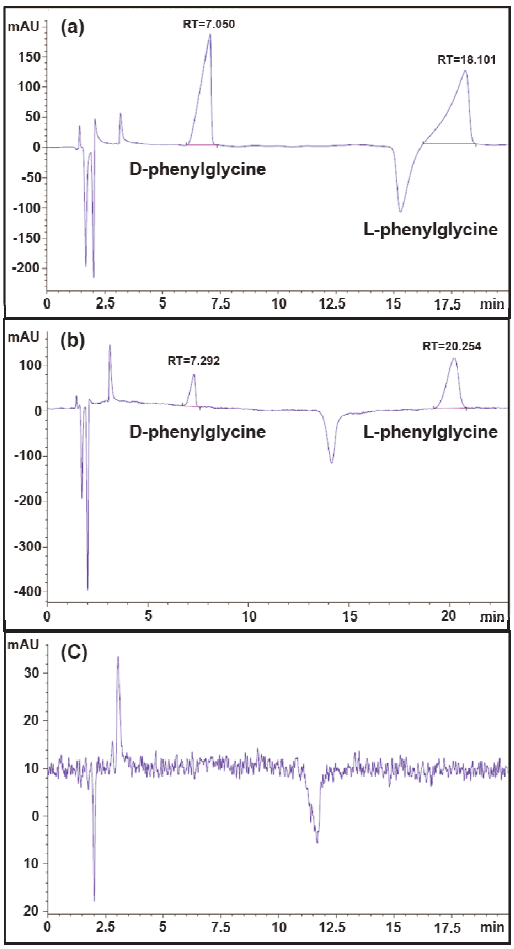


**Figure.** 2 Effect of the concentration of copper(II) and L-proline on enantioseparation, 1:2 mM (a), 2.5:5 mM (b) and 5:10 mM (c).

### Effect of pH

The pH influence on the formation of the ternary complex was studied by adjusting the pH of mobile phase to various pH (4-8) by fixing the percent organic modifier at 5% and the concentration of copper(II) to L-proline at 2.5:5 mM. It was found that the separation conducted at pH 6 gave the short time of analysis, approximately 20 min, with satisfactory characteristics of chromatograms including resolution and tailing factor (Figure. 3). Hence, it was chosen as the optimal pH.

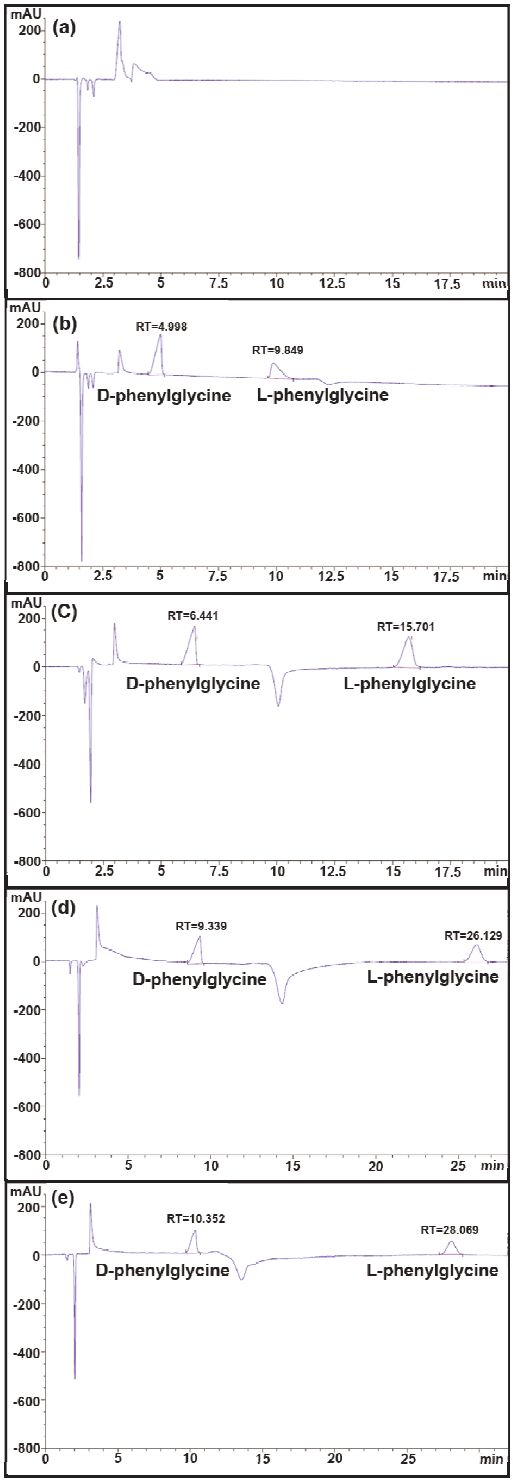


**Figure.** 3 Effect of pH on enantioseparation, pH 4 (a), pH 5 (b), pH 6 (c), pH 7 (d) and pH 8 (e).

**Effect of organic modifier in mobile phase**

In this study, methanol was used as an organic modifier to optimize the elution time. By varying the concentration of methanol in mobile phase and fixing the concentration of copper(II) to L-proline at 2.5:5 mM and pH at 6, it was found that, the resolution and retention time decreased with the increasing percent of methanol (Figure. 4). The amount of methanol in the range of 10 - 20% could be used to reduce the RT. However, to avoid the overlapping of the other peaks of possible impurities which might be adulterating in the samples e.g. by-products from the synthesis process, 10% methanol was chosen in this study.

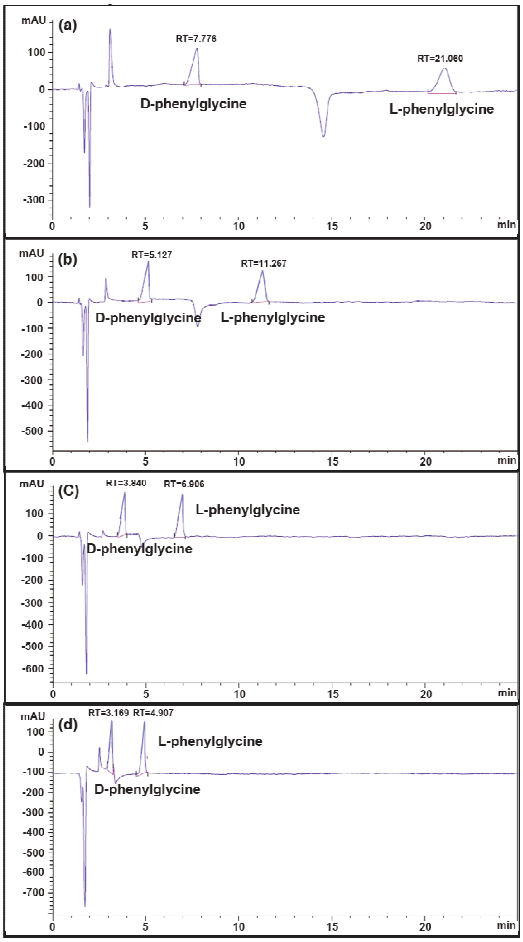


**Figure.** 4 Effect of percent of methanol on the

enantioseparation, 5% (a), 10% (b), 15% (c) and 20% (d).

**Linearity and range**

After the optimal protocols were established, the standard curves of D-PHG and L-PHG were constructed to verify the performance. An excellent linear response of peak area in relation to the concentration of D-PHG and L-PHG was observed over the range of 0.10-2.00 mM with the regression coefficient of 0.9997 and 0.9998 for D-PHG and L-PHG, respectively. The linear regression equations were A=5295.5C+260.31 for D-PHG and A=6074.3C+291.06 for L-PHG, when A is peak area (mAU) and C is concentration (mM) (Figure. 5).

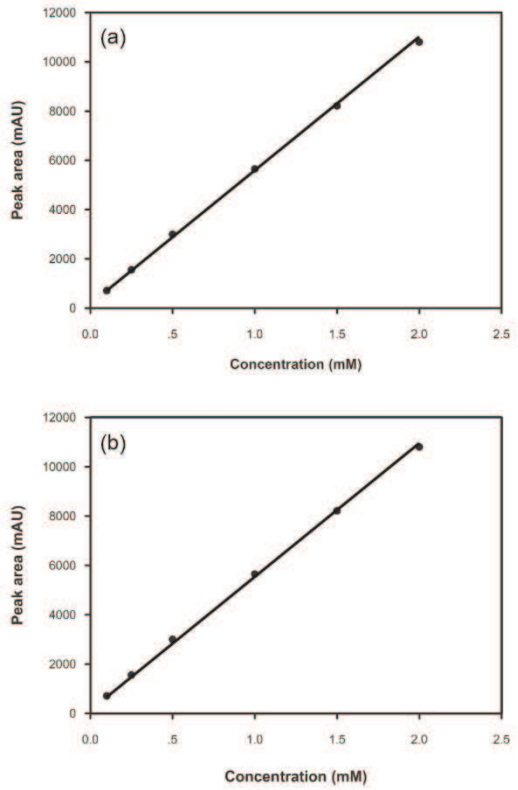


**Figure. 5** Standard curves of D-PHG (a) and L-PHG (b) from the optimal protocol.

## Discussions and Conclusion

Copper(II) complexes with L-proline can be successfully used as the chiral selectors for enantioseparation and determination of D-PHG and L-PHG by HPLC. This method, based on ligand-exchange chromatography, is facile, rapid and requires no expensive chiral columns. Due to the acceptable linearity and resolution, the optimal conditions investigated in this study can be further used for the development and validation of a quantitation method for D-PHG and L-PHG.

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