*Pharmaceutical Sciences, June 2016, 22, 76-80*
doi: 10.15171/PS.2016.13
*http://journals.tbzmed.ac.ir/PHARM*

*Research Article*

# Naphtoyl-Glycyl-Glycyl-Glycine: A New Substrate for Angiotensin Converting Enzyme (ACE) Assay Using HPLC

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**Article Info**

***Article History:***
Received: 22 September 2015
Accepted: 01 February 2016
ePublished: 30 June 2016

***Keywords:***
-Angiotensin converting enzyme
-Naphthoyl-glycyl-glycyl-glycine
-Naphthoyl-glycine
-HPLC
-Substrate
-Assay

**ABSTRACT**

***Background:*** Several in vitro assays are used to determine Angiotensin Converting Enzyme (ACE) activity. The purpose of the present investigation, was developing a practical and extraction-free chromatographic method to determine ACE activity.
***Methods:*** The method relies on UV-detection of Naphthoyl-glycine (NG), which is resulted from enzymatic hydrolysis of the synthetic substrate, Naphthoyl-glycyl-glycyl-glycine (NGGG), applying a reverse phase chromatographic separation. In this regard, experimental conditions for the assay such as Enzyme/Substrate (E/S) ratio and incubation time were optimized. Chromatographic separation was performed on a reverse phase C18 column (250 × 4.6 mm), using a mobile phase consisting of acetonitrile/water (20:80, v/v), pH = 5.0 with a flow rate of 2.0 mL/min and a detection wavelength of 280 nm.
***Results:*** The optimized Enzyme/Substrate (E/S) ratio and incubation time were 10 mU/nmol and 35 min respectively. The results indicated that the calibration curve was linear (r2 = 0.994) and the average recovery (n = 6) of NG was 99.5 ± 1.3% (mean ± RSD).
***Conclusion:*** In this study, we introduced a method which is an efficient approach to determine ACE activity due to its sensitive, accurate, and reliable performance with great repeatability.

## Introduction

Angiotensin Converting Enzyme (ACE) is a dipeptidyl carboxypeptidase (EC 3.4.15.1) which plays an important role in regulating blood pressure. ACE catalyzes the conversion of angiotensin I (Ang I) to a potent vasoconstrictor angiotensin II (Ang II).[1,2] Therefore, ACE activity leads to a considerable increasing in Ang II concentration. As a result, taking ACE inhibitors such as captopril and enalapril is one of the most popular therapeutic principles in management of hypertension and heart failure.[3-6]

It is well established that ACE is a transmembrane peptidase in various epithelial and neuroepithelial tissues such as the brush borders of placenta, kidney, intestine and choroid plexus. Moreover, ACE has been found in various areas of the brain and the male genital tract.[7,8] Therefore, there is an increasing need to monitor ACE activity using simple, economical, and practical methods. Until now, determination of ACE activity has been performed with the aid of either the physiological substrate, Ang I, or various synthetic analogues such as hippuryl-histidyl-leucine (HHL),[9] N-(3-[2-furylacryloyl)-Phe Gly-Gly (FAPGG),[10] Hip-Gly-Gly (HGG),[11] etc. The procedures have been established based on chromatographic,[10,12] spectrophotometric,[9,13,14] spectrofluorimetric[15,16] and radiochemical techniques.[17] However, these methods suffer some limitations: **(a)** tedious procedure for the synthesis of substrate is usually required, **(b)** some enzymatic products do not show sufficient and sensitive analytical signals in the assay, **(c)** expensive reagents are required, and **(d)** time consuming extraction procedures prior to the analysis are necessary in order to remove interferences and also to concentrate the analyte. Therefore, to overcome the above mentioned difficulties, novel procedures to determine ACE inhibition activity are still essential.

In the present work, NGGG was synthesized as a new substrate for the ACE assay. The analytical responses of NG as the enzymatic product and as a sign of the reaction rate were studied in detail. The ACE activity was evaluated by determining the hydrolysis

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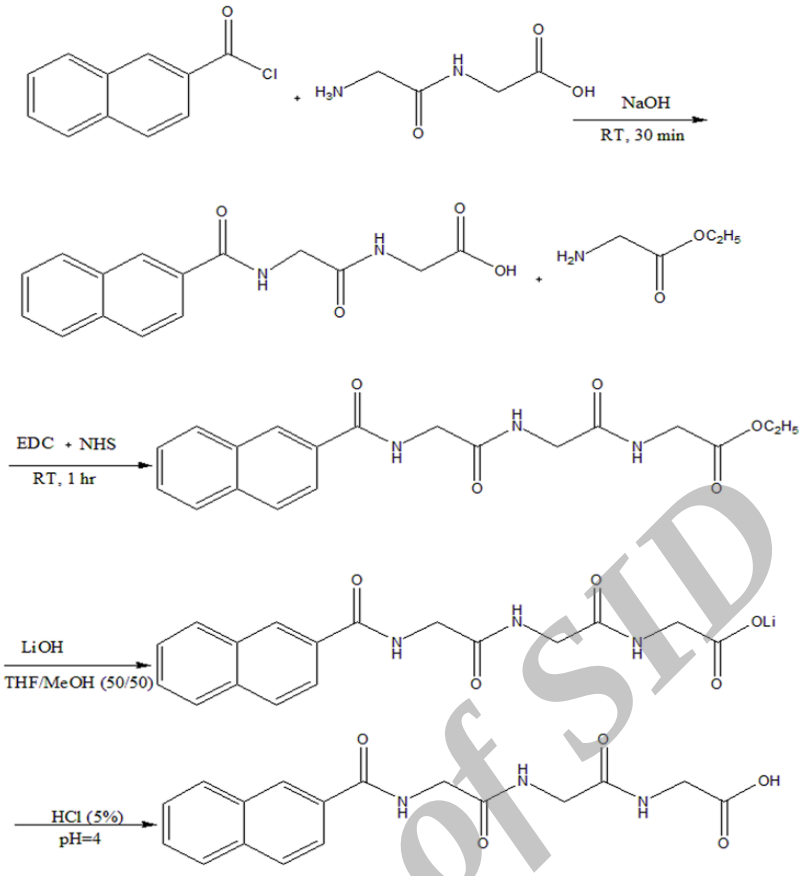


**Figure 1.** Synthesis of Naphthoyl-glycyl-glycyl-glycine (NGGG).

rate of substrate, NGGG, applying reverse phase high performance liquid chromatography (RP-HPLC) with UV detection.

## Materials and methods

### *Chemicals*

ACE from rabbit lung was purchased from Sigma Chemical Co. (England). Captopril, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), 2-naphthoyl chloride, ammonium sulfate, glycyl-glycine (gly-gly), ethyl acetate, ethyl glycinate hydrochloride, hexan, hydrochloric acid (37%), 2-propanol, tetrahydrofuran (THF), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), ethylenediaminetetraacetic acid (EDTA), lithium hydroxide, sodium hydroxide, N-hydroxysuccinimide (NHS), sodium bicarbonate, HPLC grade acetonitrile, and glacial acetic acid were purchased from Merck Co. (Germany). Deionized water was used for preparation of all aqueous solutions.

### *Apparatus*

HPLC Analysis was performed on RP-HPLC system (Knauer, Germany) with a Eurospher $C_{18}$ column (250 × 4.6 mm) which is protected by a pre-column (30 × 3.9 mm). The system was equipped with a solvent delivery system pump (Smartline pump 1000), Rheodyne sample injection valve (Model 7725i) with 20 µl sample loop, and a Smartline PDA detector 2850. Peak areas were calculated using a Chromgate software version 3.3. An Eppendorf thermomixer (Germany) was used for performing enzymatic reactions.

### *Synthesis of Naphthoyl-glycyl-glycyl-glycine*

In order to naphthoyl-glycyl-glycine (NGG) synthesis (Figure 1), gly-gly (GG) (1.5 g, 12 mmol) was dissolved in minimum amount of water. Then sodium hydroxide solution (10 mmol) and 2-naphthoyl chloride (2.28 g, 12 mmol) was gradually added to the solution, followed by stirring at room temperature for 30 min. After the end of the reaction, concentrated HCl (5 mL) was added to the reaction mixture and the precipitated product was separated by filtration. The crude product of NGG was recrystallized from 2-propanol and characterized by spectroscopic methods.

In the next step, the mixture of NGG (1 eq), EDC (1.1 eq) and NHS (1 eq) in acetonitrile were reacted at room temperature for 1 h. Then ethyl glycinate (which had been reacted with sodium bicarbonate 15% solution) was added to the reaction mixture and stirred for additional 2 h. The precipitated product was filtered and the pure product, NGGG was obtained by recrystallization from 2-propanol.

At the final step, the latter product was hydrolyzed by LiOH in THF/MeOH (1:1).

After the end of the reaction, the aqueous solution of HCl (5%) was added dropwise in order to obtain the pH = 4. Then the solvent was evaporated under reduced

pressure. The obtained product was analytically pure and the structure was confirmed by, $^1$H NMR, IR and MASS spectroscopy. Yield: 85%; mp 331-333 °C; IR (KBr) cm$^{-1}$ 2500-3500, 3412, 1682, 1600; $^1$H-NMR (DMSO-$d_6$): 3.73 (d, 2H, $CH_2$, *J* = 5.6 Hz), 4.05 (d, 2H, $CH_2$, *J* = 5.6 Hz), 4.38 (s, 2H, $CH_2$), 7.30 (t, 1H, NH, *J* = 8.1 Hz), 7.58-7.63 (m, 2H, ArH), 7.97-8.04 (m, 4H, ArH), 8.51 (t, 1H, NH, *J* = 5.6), 8.55 (s, 1H, ArH), 9.18 (t, 1H, NH, *J* = 5.6); Ms (m/z) = 343.

### *Synthesis of 2-naphthoyl-glycine (Naph-gly)*

The Naph-gly was prepared according to a literature procedure.[18] Briefly, 2-naphthoyl chloride (0.57 g, 3.03 mmol) and glycine (0.25 g, 3.03 mmol) were stirred in the presence of NaOH (0.12 g, 3.03 mmol) in aqueous $CH_3CN$ ($CH_3CN$/ $H_2O$ : 2mL/ 5mL) at room temperature. After 1 h, the organic solvent was evaporated, washed with aqueous HCl (6 M), and extracted with EtOAc. The organic layer was then washed with brine, dried over $MgSO_4$ and recrystallized from EtOAc/hexane to yield NG as white cocrystals (0.48 g, 68 %); mp 150.0-151.0 °C.

### *Determination of ACE activity using Naphthoyl-glycyl-glycyl-glycine as the substrate*

To 25 µL of HEPES buffer (5 mM HEPES and 300 mM NaCl; pH=8.3), 25 µL of ACE solution (80 mU/mL) was added and then preincubated at 37 ºC for 3 min. After that, 25 µL of substrate solution (9 µM) was added to the mixture to start the reaction. After 35 min incubation at 37 ºC, the reaction was stopped by adding 50 µL of EDTA solution (0.93 g EDTA and 23.83 g HEPES; pH=9). Then the mixture was filtered through a 0.2 m filter prior to application to the HPLC column.

The mobile phase was an isocratic system consisting of acetonitrile/water (20:80, v/v). The pH of the mobile phase was adjusted to 5.0 by adding 1.0 mL glacial acetic acid per liter of the mixture. The injection volume was 20 µL and the flow rate was 2.0 mL/min. The final hydrolyzed product (NG) was detected at 280 nm (Figure 2). In the presence of the inhibitor (captopril), 25 µL of the inhibitor solution was used instead of 25 µL of HEPES buffer solution. To determine the $IC_{50}$ value, 25 µL of HEPES buffer solution was replaced with 25 µL of captopril solution in different concentrations in the assay procedure.

### *ACE activity measurement*

The percent of ACE activity could be calculated according to the equation below:

$$\text{ACE inhibition}\ (\%) = \left[1 - \left(\text{AUC}_{\text{inhibitor}}/\text{AUC}_{\text{control}}\right)\right] \times 100$$

Eq.(1)

Where $\text{AUC}_{\text{inhibitor}}$ and $\text{AUC}_{\text{control}}$ are the Area Under Curve of the NG peak in the presence of inhibitor and that of the control sample (absence of the inhibitor), respectively.

## Results and Discussion

### *Optimization of the HPLC conditions*

In order to optimize the chromatographic conditions, several experiments were performed under different conditions including acetonitrile/water (20:80, v/v) at pH 5.0 as the mobile phase and the flow rate of 2.0 mL/min. The retention times were approximately 5.3 and 7.3 min for NGGG and NG, respectively.

### *Calibration curve for Naph-gly standard solutions*

Different concentrations of NG (0.05, 0.15, 0.30, 0.45, 0.60 mM) were injected into the HPLC system and the calibration curve was evaluated. In this range, the analytical signals indicated good linearity ($r^2$ = 0.994). The average recovery (n = 6) of 0.45 mM NG was 99.5% with relative standard deviation (RSD), 1.3%.

### *Choice of substrate*

In general, choosing the substrate in the assay is an important step in order to evaluate the enzymatic activity. For the best selection, different points should be considered: (i) sensitivity, (ii) time of analysis, and (iii) cost of the reagents used for the synthesis of substrate. NGGG as a new substrate was cleaved by the enzyme into NG and GG and the resulted product, NG, was analyzed by RP-HPLC. NG and the unreacted substrate was separated under the chromatographic conditions with retention times of 5.3 and 7.3 min for NGGG and NG, respectively.

### *Optimal conditions for the enzymatic assay*

The reaction rate is dependent on some parameters such as Enzyme/Substrate (E/S) ratio and incubation time. Therefore, the above mentioned parameters were optimized for ACE assay of the new substrate.

### *E/S ratio*

Regarding to E/S ratio optimization, different ratios of E/S were examined with incubation time of 30 min (0.6, 3, 9, 15 mU/nmol E/S). The results confirm that the ratio of 10 mU/nmol is the optimum ratio of the E/S for the substrate NGGG.

### *Optimization of incubation time*

To optimize the incubation time and repeatable reaction results, various incubation times (15, 25, 35, 45, 60 min) were examined using the optimum E/S ratio (10 mU/nmol). Therefore, 35 min was selected as an optimum reaction time for hydrolysis of substrate (NGGG) by ACE (Figure 3).

### *Determination of ACE-inhibition activity of captopril and calculation of IC50*

The $IC_{50}$ value of captopril was determined from dose-response curve using Graphpad software, Prism 5. In this way, $IC_{50}$ value for captopril was 15 nM. The results obtained in this experiment were summarized in Table 1 and Figure 4.

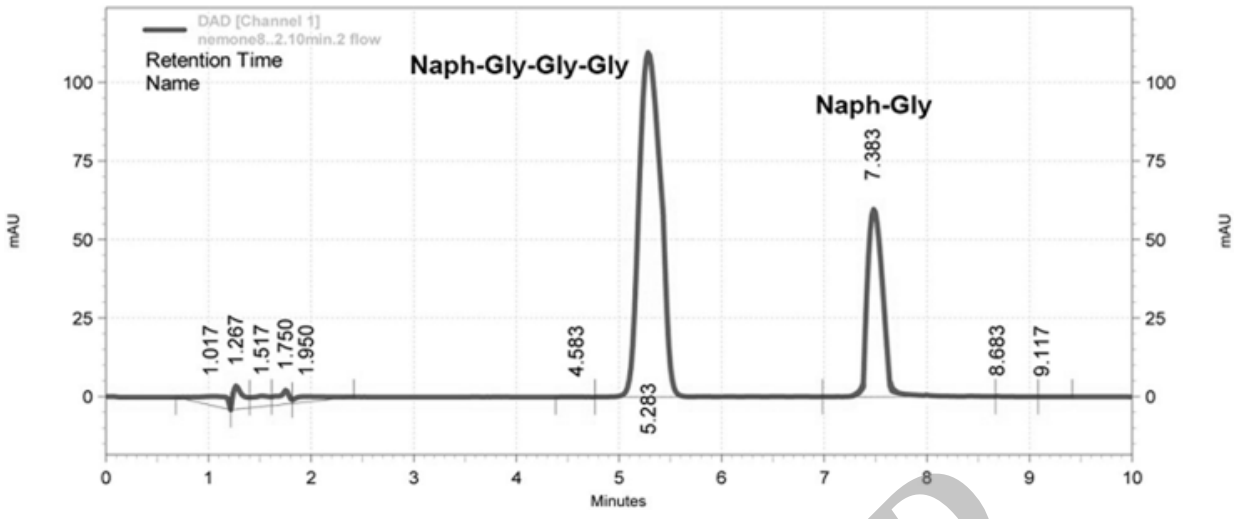


**Figure 2.** Typical HPLC chromatogram of the enzymatic hydrolysis of naphthoyl-glycyl-glycyl-glycine (NGGG) and its product naphthoyl-glycine (NG).

**Table 1.** Determination of ACE inhibition activity of captopril ($IC_{50}$).

| Concentration of captopril (nM) | Inhibitory activity (% ± SD) * |
|---|---|
| 4 | 22 ± 0.79 |
| 8 | 34 ± 0.85 |
| 15 | 50 ± 0.95 |
| 30 | 76 ± 1.63 |
| 60 | 90 ± 1.56 |

* Values are means ± SD of three measurements.

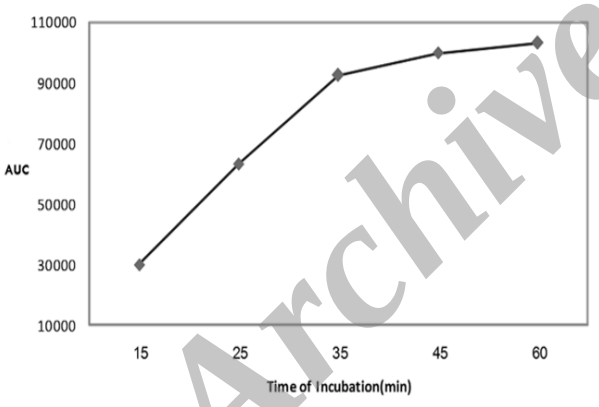


**Figure 3.** Optimization of incubation time of ACE and Naphthoyl-glycyl-glycyl-glycine (NGGG) as substrate.

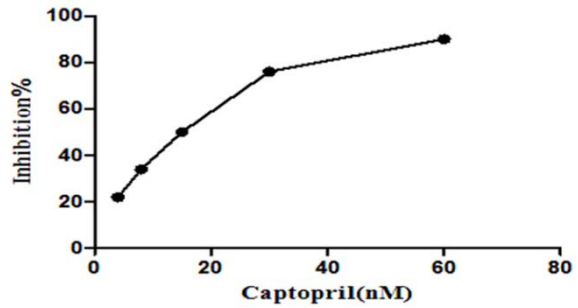


**Figure 4.** Optimization of incubation time of ACE and Naphthoyl-glycyl-glycyl-glycine (NGGG) as substrate.

## Conclusion

In this work, NGGG was synthesized as a new substrate for ACE activity determination applying HPLC. The substrate and the hydrolyzed product were separated under optimized conditions with retention times 5.3 and 7.3 min for NGGG and NG, respectively. Under the optimum experimental conditions, E/S ratio was defined as 10 mU/nmol and incubation time as 35 min. The sensitivity of the assay was confirmed through obtained $IC_{50}$ value of 15 nM for captopril. These results show a good improvement in performing ACE assay in comparison with previous reported ones in terms of sensitivity of the assay, absence of interferences, reagent costs and the easy substrate synthetic procedure. The obtained results revealed that the new substrate and developed method is an efficient approach for determination ACE activity due to its sensitive, accurate, inexpensive and reliable performance.

## Acknowledgments

The authors would like to thank the Research Council of Tehran University of Medical Sciences for providing the financial support for this work.

## Conflict of interests

The authors claim that there is no conflict of interest.

## References

1. Cabello-Verrugio C, Córdova G, Salas JD. Angiotensin II: Role in skeletal muscle atrophy. Curr Protein Pept Sci. 2012;13(6):560-9.

doi:10.2174/138920312803582933
2. Rao S, Sun JB, Liu Yb, Zeng HC, Su YB, Yang Y. ACE inhibitory peptides and antioxidant peptides derived from in vitro digestion hydrolysate of hen egg white lysozyme. Food Chem. 2012;135(3):1245-52. doi:10.1016/j.foodchem.2012.05.059
3. Qin DS, Liu X, Liu GD. New Concept on Renin angiotensin System. Chinese J Urology. 1996;17:50-4.
4. Unger T. The role of the renin-angiotensin system in the development of cardiovascular disease. Am J Cardiol. 2002;89(2):3-9. doi:10.1016/S0002-9149(01)02321-9
5. Coates D. The angiotensin-converting enzyme (ACE). Int J Biochem Cell Biol. 2003;35:769-73. doi:10.1016/S1357-2725(02)00309-6
6. Sharifi N, Souri E, Ziai SA, Amin GH, Amini M, Amanlou M. Isolation, Identification and Molecular Docking Studies of a New Isolated Compound, from *Onopordon acanthium*: A Novel Angiotensin Converting Enzyme (ACE) inhibitor. J Ethnopharmacol. 2013;148(3):934-939. doi:10.1016/j.jep.2013.05.046
7. Ganong WF. The brain renin-angiotensin system. Annu Rev Physiol. 1984;46:17-31. doi: 10.1146/annurev.ph.46.030184.000313
8. Erdos EG, Skidgel RA. The unusual substrate specificity and the distribution of human angiotensin I converting enzyme. Hypertension. 1986;8(4):1134-7.
9. Hayakari M, Kondo Y, Izumi H. A rapid and simple spectrophotometric assay of angiotensin converting enzyme. Anal Biochem. 1978;84(2):361-9. doi:10.1016/0003-2697(78)90053-2
10. Lahogue V, Rehel K, Taupin L, Haras D, Allaume P. A HPLC-UV method for the determination of angiotensin I converting enzyme (ACE) inhibitory activity. Food Chem. 2010;118(3):870-5. doi:10.1016/j.foodchem.2009.05.080
11. Serra CP, Cortes SF, Lombardid JA, Braga de Oliveira A, Brag FC. Validation of a colorimetric assay for the in vitro screening of inhibitors of angiotensin-converting enzyme (ACE) from plant extracts. Phytomedicine. 2005;12(6-7):424-32. doi:10.1016/j.phymed.2004.07.002
12. Pan MJ, Zhao YC, Mao SL. Effect of Dilong on ACE Activity of Rats. Zhejiang J Integr Trad Chin West Med. 2006;16(11):667-83.
13. Cushman DW, Cheung HS. Spectrophotometric assay and properties of the angiotensin converting enzyme of rabbit lung. Biochem Pharmacol. 1971;20(7):1637-48. doi:10.1016/0006-2952(71)90292-9
14. Neels HM, Sande Van ME, Scharpe SL. Sensitive colorimetric assay for angiotensin converting enzyme in serum. Clin Chem. 1983;29(7):1399-403.
15. Friedland J, Silverstein EA. Sensitive fluorimetric assay for serum angiotensin converting enzyme. Am J Clin Pathol. 1976;66(2):416-424.
16. Friedland J, Silverstein E. Sensitive fluorimetric assay for serum angiotensin converting enzyme with the natural substrate angiotensin I. Am J Clin Pathol. 1977;68(2):225-8. doi:10.1093/ajcp/68.2.225
17. Rohrbach MS. [Glycine-1-$^{14}$C] hippuryl-L-histidyl-L-leucine: a substrate for the radiochemical assay of angiotensin converting enzyme. Anal Biochem. 1978;84(1):272-6. doi:10.1016/0003-2697(78)90510-9
18. Katritzky AR, Yoshioka-Tarver M, El-Gendy BEGM, Hall CD. Synthesis and photochemistry of pH-sensitive GFP chromophore analogs. Tetrahedron Lett. 2011;52(17):2224-7. doi:10.1016/j.tetlet.2010.12.082