

Determination of angiotensin converting enzyme inhibitory activity by high-performance liquid chromatography/electrospray-mass spectrometry

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Abstract

A sensitive and rapid method for determination of angiotensin converting enzyme (ACE) inhibitory activity was developed based on a combination of enzymatic reaction followed by high performance liquid chromatography/electrospray-mass spectrometry (HPLC-ESI-MS) determination of its product. The most commonly used substrate hippuryl-histidyl-leucine (HHL) or hippuryl-glycyl-glycine (HGG) hydrolysis catalyzed by purified rabbit lung ACE or human plasma ACE was investigated in the presence of benazeprilat. The incubation time was 8 min for purified lung ACE, and 16 min for human plasma ACE. The produced hippuric acid (HA) was separated from substrate HHL or HGG by HPLC on a C₁₈ column with isocratic elution within 6.5 min, and quantified by electrospray ionization mass spectrometry (ESI-MS) with *p*-phthalic acid as an internal standard (IS). The limit of detection of HA was 6.0 ng/ml. HHL or HGG hydrolysis catalyzed by purified lung ACE displayed excellent accuracy and reproducibility. The small total reaction volume, the low concentration of substrate, and the simple treating procedures present the advantages of the new method. Furthermore, the total time of the whole procedure for one sample with the novel method is less than 1/2 of that of the conventional HPLC or spectrophotometry method, while the accuracy and the precision of the new method are almost the same as the conventional HPLC method with UV detection.

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1. Introduction

Angiotensin converting enzyme (ACE, peptidyl dipeptide hydrolase, EC 3.4.15.1) plays a key physiological role in the renin-angiotensin system to increase blood pressure, which removes a dipeptide from the C terminus of angiotensin I to form angiotensin II, a potent vasoconstrictor, and inactivates the vasodilator nonapeptide bradykinin [1,2]. The inhibition of ACE is considered to be an important therapeutic approach in the treatment of high blood pressure, and ACE inhibitors have become the preferred class of antihypertensive agents for the treatment of patients with concurrent secondary diseases [3,4].

Many synthetic ACE inhibitors such as benazepril, captopril, enalapril, alacepril and so on are currently used in the treatment of hypertension and heart failure. Although most of the big studies have proved the great beneficial effects of these agents, some of ACE inhibitors also exhibit a low incidence of adverse side effects such as cough, skin rashes and so on [5–7]. Recently, many studies have been carried out to discover ACE inhibitory compounds such as peptides, proteins, heterochitooligosaccharides [8] and so on from various natural sources. And because of the abundances of natural resources, the research for natural ACE inhibitors as alternatives to synthetic ones is of great interest among researchers for safe and economical use [8].

To study ACE inhibitor, a simple, rapid, and reliable method for monitoring of ACE inhibitory activity *in vitro* is important. *In vitro*, ACE inhibitory activity is commonly obtained by monitoring the concentration of product formed by causing appropriate substrate to react with the ACE in the presence and absence of

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inhibitor. Up to now, many methods such as spectrophotometry [9–11], HPLC [12–14], fluorimetry [15,16], micellar electrokinetic chromatography [17], biochemical detection [18] have been developed to determine ACE inhibitory activity. Among the methods, spectrophotometry method and HPLC method are employed most frequently. However, both spectrophotometry method and HPLC method need a relatively longer reaction time (30–80 min) [9–14,19]. Moreover, ethyl acetate extraction procedure in spectrophotometry method can also extract unhydrolyzed HHL apart from HA, therefore incorrect positive results cannot be excluded [19].

HPLC-ESI-MS as an analytical tool has provided specific quantification of compounds at much lower concentrations than conventional HPLC [20,21]. Moreover, the accuracy and reproducibility of the technique makes it well suited for the quantitative study of enzymatic-catalyzed reactions that does not require a chromophore or radiolabelling [22–24]. To our knowledge, assay of ACE inhibitory activity by HPLC-ESI-MS has not yet been reported. The aim of this work was to develop an alternative rapid and sensitive HPLC-ESI-MS method for determination of ACE inhibitory activity by means of the substrate HGG or HHL hydrolysis catalyzed by purified rabbit ACE or human plasma ACE, which have been used frequently for measurement of ACE activity by spectrophotometry [25–29] and HPLC method [30–33].

2. Experimental

2.1. Reagents

Benazeprilat and HA were products of the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). ACE (angiotensin converting enzyme, EC 3.4.15.1) from rabbit lung, HGG and HHL were purchased from sigma Chemical Co. (St. Louis, MO). Internal standard (*p*-phthalic acid) was product of the Tianjing Guangfu Fine Chemical Research Institute. HPLC-grade acetonitrile and methanol were obtained from Tedia Inc. (Fairfield, OH, U.S.A). Other reagents were of analytical-grade.

Double distilled water was prepared by a Millipore Milli-Q purification system (Millipore Corp. Bedford, MA, USA). Fresh heparinized plasma was obtained from Changsha blood Center and stored below -40°C until used for analysis. (The experiment has achieved the approval of Hunan Health and Drug Administration.) Buffer A, 100 mM Tris-formic acid buffer (pH 8.3) containing 300 mM NaCl [9], and Buffer B, 100 mM Tris-formic acid buffer (pH 8.0) containing 100 mM NaCl [34], were prepared fresh daily.

2.2. HPLC-ESI-MS conditions

HPLC was performed on a Waters (Milford, MA, USA) Alliance 2695 liquid chromatographic system equipped with a 996 photodiode array detector. Separations were accomplished on a Johnson Spherigel C₁₈ column (250 mm \times 4.6 mm, 5 μm , Dalian, China) and the column temperature was maintained at 30°C . Substrate, HA, and IS were detected at 228 nm. The

mobile phase consisted of acetonitrile and 0.08% acetic acid in water, delivered at a constant flow rate of 0.2 ml/min for acetonitrile and 0.8 ml/min for 0.08% acetic acid aqueous solution. The mobile solution was split 1:3 postcolumn into the mass spectrometer. A 10- μl aliquot of the treated example was injected for analysis.

A Micromass ZQ 2000 ESI mass spectrometer, in selected ion monitoring mode, was used for quantitative mass spectrometric detection. The mass spectrometer was operated in the negative mode to generate $[\text{M}-\text{H}]^{-}$ ions at m/z 178.5 for HA, and m/z 165.5 for IS; the dwell time was 0.3 s both for HA and IS. The capillary voltage was 3500 V, the cone voltage was 23 V, the extractor voltage was 5 V, and the RF lens voltage was 0.5 V. The ionization resource was worked at 120°C , and the desolvation temperature was set at 270°C . Nitrogen, at a flow rate of 300 l/h and 60 l/h, was used as desolvation and cone gas, respectively. Instrument control, data acquisition and analysis were performed using the MasslynxTM 4.0 data system software.

2.3. Calibration curve

To construct a calibration curve, a series of standard HA solutions were prepared (0.08–80 $\mu\text{g}/\text{ml}$) with 100 mM Tris-formic acid buffer (pH 8.0) as solvent. The calibration was constructed in triplicate to 8 different concentrations. Then all the HA standard solutions were spiked with different volume of 6 $\mu\text{g}/\text{ml}$ IS prior to the HPLC-ESI-MS analysis. Calibration curve was obtained by plotting the relative peak-area ratio of the HA to IS, versus the concentration of HA.

2.4. Sample preparation with the purified rabbit lung ACE

For substrate HHL or HGG hydrolysis catalyzed by purified rabbit ACE, the total reaction volume was 80 μl . Buffer A was used to dilute the substrate HHL, corresponding assay sample and rabbit lung ACE; Buffer B was used to dilute the substrate HGG, corresponding assay sample, and purified rabbit lung ACE. To 20 μl enzyme solution (2 mU), 20 μl assay sample solution and 40 μl 4 mM substrate HHL or HGG were added, while the control reaction mixture contain 20 μl buffer instead of assay sample. After 8 min incubation at 37°C in a water bath, the reaction was terminated by the addition of 160 μl of 2.0 $\mu\text{g}/\text{ml}$ IS acetonitrile solution, and then the solution was filtered through a 0.45 μm filter for analysis.

2.5. Sample preparation with human plasma ACE

For substrate HHL or HGG hydrolysis catalyzed by human plasma ACE, the total reaction volume was 100 μl . Buffer A was also used to dilute the substrate HHL and assay sample; buffer B was used to dilute the substrate HGG and assay sample. To 40 μl of human plasma containing endogenous ACE, 10 μl assay sample solution and 50 μl 4 mM HHL or HGG were added, while the control reaction mixture contain 10 μl buffer instead of assay sample. After 16 min incubation at 37°C in a water bath, the reaction was terminated by the addition of 200 μl of 2.0 $\mu\text{g}/\text{ml}$ IS acetonitrile solution. After vortex shaking for 5–8 s,

all the samples were then centrifuged at 1.289×10^4 g for 8 min at 4 °C and the clear supernatant of each sample was for analysis.

ACE inhibition rate was calculated by the equation:

ACE inhibition rate (%)

$$= 100 \times \left\{ \frac{([\text{HA}]_c/[\text{IS}] - [\text{HA}]_s/[\text{IS}])}{([\text{HA}]_c/[\text{IS}])} \right\}$$

where $[\text{HA}]_c/[\text{IS}]$ is the concentration ratio of the HA in control reaction to IS, $[\text{HA}]_s/[\text{IS}]$ the concentration ratio of the HA in sample reaction to IS.

3. Results and discussion

3.1. Internal standard

To quantitatively monitor the HA, one of the products of ACE reacting with HHL or HGG, by ESI-MS, the choice of an IS for HA is necessary. Although the ideal IS should be the isotopically labeled HA, commercial stable-isotope-labeled HA was not available. Therefore, we have to select the IS based on the similarity of molecular structure, ionization pattern and retention time with the HA. A number of compounds, such as benzoic acid, *p*-phthalic acid, *p*-nitrobenzoic acid and so on were investigated. The *p*-phthalic acid was satisfactory to act as the IS of HA because of its similar retention time on C₁₈ column (see Fig. 1) and good response of ESI-MS in negative ion mode.

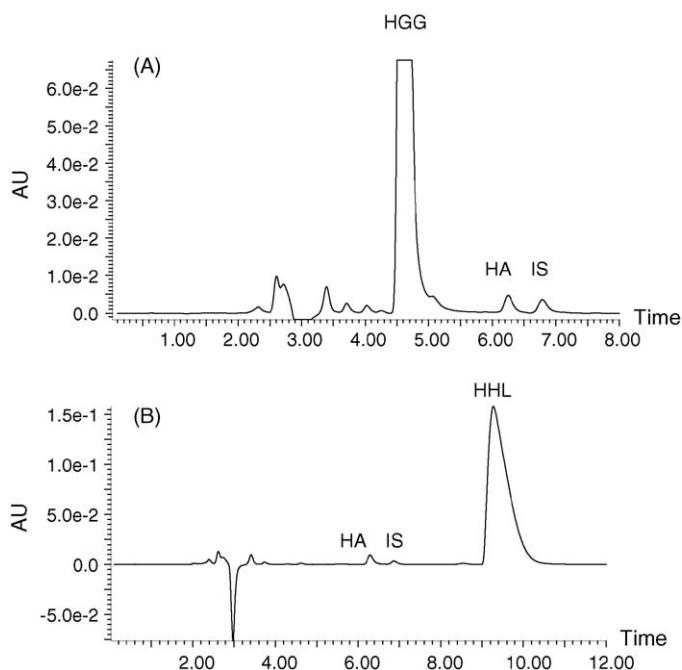


Fig. 1. HPLC chromatograms for separation of reaction mixture with human plasma as the source of ACE: (A) 0.2 mM HGG hydrolysis mixture catalyzed by plasma ACE for 16 min in buffer B, spiked with 1.6 $\mu\text{g}/\text{ml}$ IS; (B) 0.2 mM HHL hydrolysis mixture catalyzed by plasma ACE for 16 min in buffer A, spiked with 1.6 $\mu\text{g}/\text{ml}$ IS.

3.2. HPLC and ESI-MS conditions

Representative chromatograms of human plasma ACE reaction mixture with *p*-phthalic acid as IS are provided in Fig. 1. Under the aforementioned HPLC conditions, complete baseline separation of HA and HGG or HHL was achieved on a C₁₈ column. HA and IS were eluted at 6.3 min and 6.8 min, respectively. A better peak shape of HHL could also be achieved with an optimal gradient elution, and the total time for the separation of HHL or HGG, HA, and IS was almost the same with both the optimal gradient and isocratic elution. However, the gradient elution needed at least 5 min to equilibrium the column for the next injection, and the signal-to-noise (S/N) ratio of HA was not significantly improved. So the optimal isocratic elution condition was time-saving and practical.

To obtain an optimum response of mass spectrometry, three commonly used polar solvents for reversed-phased HPLC-ESI-MS (water, acetonitrile, and methanol) and three kinds of volatile, and thus compatible with the ESI-MS, mobile phase modifiers (trifluoroacetic, acetic, and formic acid) were examined. A good separation of ACE reaction mixture could also be achieved by an isocratic elution of 0.35 ml/min methanol and 0.65 ml/min 0.08% acetic acid in water. However, both the HA and IS showed a better MS sensitivity in acetonitrile than in methanol. Although the addition of volatile organic acid in the mobile phase could effectively improve the tailing of HA and IS peaks and gave better separation of HA and IS, it was found that each of the three volatile acids also resulted in obvious decrease of ESI-MS response. As the concentration of each acid increased, the signal of HA and IS decreased. Among the acids, trifluoroacetic acid caused a dramatic ion suppression in the ESI, whereas with acetic acid the ion suppression was limited. The effect of ion suppression on HA increased according to the order: trifluoroacetic acid > formic acid > acetic acid, which was identical to the order of acidity. Based above-mentioned comparison, a good compromise between separation and sensitivity was made to adopt the optimum isocratic elution condition aforementioned.

Capillary and cone voltages were optimized by flow injection analysis by injection of 10 $\mu\text{g}/\text{ml}$ HA standard solution, for these two voltage parameters could greatly affect the detection sensitivity of ESI-MS. With the increase of the capillary voltage from 2000 V to 3500 V, the S/N ratios of the total ion current were also increased, and then almost reached a plateau over the range of 3500–4000 V. So we adopted the capillary voltage of 3500 V. In contrast, the S/N ratios of the total ion current would decrease when the relative lower cone voltages (below 15 V) or higher cone voltage (over 70 V) were set. The most favorable cone voltage was found to be 23 V. At cone voltage 23 V, the ESI mass spectra are characterized by pseudomolecule ions, with fragment ions of low abundance.

3.3. Calibration curve, limit of detection, limit of quantification and repeatability

To monitor the ACE reaction by ESI-MS effectively, selected ion monitoring was used to generate a calibration curve for HA

Table 1
Inter- and intra-day precision of the method

Measurement	Benazeprilat (ng/ml)	Hydrolysis of HHL catalyzed by plasma ACE		Hydrolysis of HHL catalyzed by purified rabbit ACE		Hydrolysis of HGG catalyzed by plasma ACE		Hydrolysis of HGG catalyzed by purified rabbit ACE	
		ACE inhibition rate	Precision R.S.D. ^a (%)	ACE inhibition rate	Precision R.S.D. ^a (%)	ACE inhibition rate	Precision R.S.D. ^a (%)	ACE inhibition rate	Precision R.S.D. ^a (%)
Inter- day	0.2	26.15	5.36	23.95	4.47	26.49	3.61	17.28	4.31
	0.8	59.64	7.67	62.81	2.68	68.84	5.28	64.71	1.69
	6.4	81.83	3.37	89.92	1.21	86.76	1.17	86.98	0.95
Intra- day	0.2	25.77	6.91	24.65	5.31	27.63	3.54	16.57	4.35
	0.8	57.44	11.18	61.18	2.77	71.23	5.49	64.53	1.57
	6.4	82.16	4.77	91.00	0.69	85.82	1.05	87.93	1.16

^a R.S.D.%, relative standard deviation percentage ($n=3$).

using *p*-phthalic acid as an IS. The calibration showed a linear behavior over the range 0.08–80 $\mu\text{g/ml}$ ($y = 1.0562x - 0.1343$, $R^2 = 0.9991$). The limit of detection (S/N ratio of 3:1) of HA was 6.0 ng/ml and the limit of quantification (S/N ratio of 10:1) was 20.0 ng/ml. In order to determine the repeatability of the electrospray mass spectrometer, 8.0 $\mu\text{g/ml}$ HA solution was repeatedly injected onto the C_{18} column for ten times, the relative standard deviation of peak-area ratio of the HA to the IS was only 1.52%, and the retention time were excellently in agreement with each other among the repeated runs for HA and IS. The inter-day and intra-day assay precision was also investigated by determination of ACE inhibitory rates in the presence of benazeprilat at three levels. As we can see from Table 1, The inter-day and intra-day R.S.D. (%) for purified rabbit ACE was below 4.47%, 5.31%, and for human plasma ACE 7.67%, 11.18%, respectively.

3.4. Incubation procedure

Because of the high sensitivity of ESI-MS for HA, the incubation time could be greatly shortened. With HHL or HGG as substrate, and purified rabbit lung ACE as the source of ACE, the incubation was carried out for 8 min. However, the reaction mixture must be incubated at least for 16 min with human plasma as the source of ACE, as described in the text. Blank human plasma often contains trace level of HA, which is not noticeable in our procedure by HPLC with ultraviolet detection because of its higher limit of detection, but the endogenous HA can be detected by ESI-MS (Fig. 2). To overcome this shortcom-

ing, a relatively long incubation time was adopted to control the interference of endogenous HA within 2%. Compared to the previous method based on the substrate of hippuryl dipeptides HGG or HHL, the incubation time in the new method was approximately one-fourth of that of the conventional screening method using HPLC-UV or spectrophotometer. In the literature, the incubation time of HPLC or spectrophotometry method was about 35–60 min [9–14] for purified ACE, over 60 [35,36] min for human plasma ACE. The advantage of reduced incubation time mainly benefits from the much lower limit of quantification of ESI-MS than HPLC with ultraviolet detection. Furthermore, a shorter incubation time can ensure a limited substrate hydrolysis within 10%, within which the Michaelis constant can be calculated accurately by measuring the initial velocities [23].

The stabilities of the reaction mixtures stopped by acetonitrile, 1 M HCl, and methanol were summarized in Table 2. The reaction mixtures terminated by acetonitrile demonstrate less difference and less fluctuate in the mean response of the ratio of HA to IS than those stopped by 1 M HCl or methanol at 0 °C, which might be due to the better deproteination effect of acetonitrile than that of methanol, and substrate hydrolysis catalyzed by hydrochloride acid. Therefore, acetonitrile was adopted to terminate all the reaction mixture. And these treated reaction mixture could be stable up to 5 h at 0 °C with the difference from the initial peak-area ratio of HA to IS not more than 3.7%.

3.5. Comparison of four different ACE activity assays

The newly developed HPLC-ESI-MS method was used to screen for the activity of ACE in the presence of benazeprilat. Table 3 provided the ACE inhibition rate values of benazeprilat

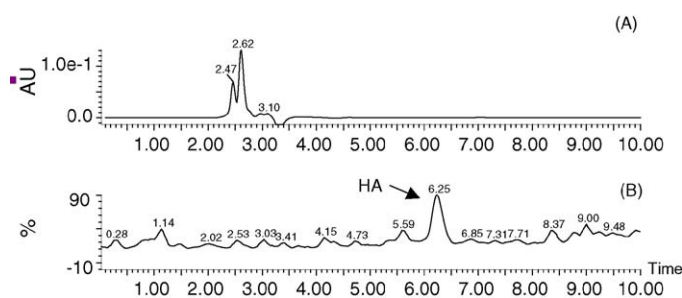


Fig. 2. HPLC-ESI-MS analysis of blank human plasma: (A) HPLC profile detected at 228 nm; (B) corresponding selected-ion current profile detected at m/z 178.5. Sample treatment: to 40 μl human plasma, 260 μl acetonitril was added, after centrifugation, 10 μl supernatant was for analysis.

Table 2

The stability of the incubation mixtures treated by acetonitrile, 1 M HCl, and methanol in the refrigerator 5 h at 0 °C

Incubation mixtures	Percent Difference from the initial peak-area ratio of HA to IS		
	By acetonitrile	By 1 M HCl	By methanol
HGG and plasma ACE	-0.600–3.52	1.26–5.95	2.82–5.30
HGG and purified ACE	1.175–3.16	2.09–5.29	-1.00–4.67
HHL and plasma ACE	2.61–3.74	-1.81–6.29	3.47–6.51
HHL and purified ACE	-0.69–2.55	2.00–5.87	-0.84–4.83

Table 3
ACE inhibitory activities of benazeprilat obtained by four different assays

Concentration of benazeprilat(ng/ml)	ACE inhibition rate (%) ^a			
	Hydrolysis of HHL catalyzed by plasma ACE	Hydrolysis of HHL catalyzed by purified rabbit ACE	Hydrolysis of HGG catalyzed by plasma ACE	Hydrolysis of HGG catalyzed by purified rabbit ACE
0.2	25.77 ± 1.78	24.65 ± 1.31	27.63 ± 0.98	16.57 ± 0.72
0.4	37.12 ± 2.23	43.86 ± 1.87	47.16 ± 5.15	32.84 ± 1.39
0.8	57.44 ± 6.42	61.18 ± 1.70	71.23 ± 3.91	64.53 ± 1.13
1.6	72.35 ± 3.12	78.70 ± 3.11	78.96 ± 1.57	78.65 ± 0.38
3.2	76.68 ± 6.22	83.72 ± 2.06	80.05 ± 1.97	80.66 ± 1.16
6.4	82.16 ± 3.92	91.00 ± 0.63	85.82 ± 0.90	87.93 ± 1.02
12.8	91.74 ± 1.12	96.21 ± 0.05	96.77 ± 1.45	94.49 ± 0.26

^a $n = 3$, mean ± standard deviation.

lat over the range 0.2–12.8 ng/ml by four different assays, i.e. HHL or HGG hydrolysis catalyzed by purified rabbit lung ACE or human plasma ACE. Typical HPLC-ESI-MS chromatograms for these four assays in the presence of 0.8 ng/ml benazeprilat are shown in Figs. 3 and 4. Although the values showed slightly

a little difference between the four different assays at the same concentration of benazeprilat, owing to the differences of substrate, sources of enzymes or buffer solution [37], the overall inhibitory trends of benazeprilat were very similar. As we can see from Table 3, on the whole, the standard deviations derived

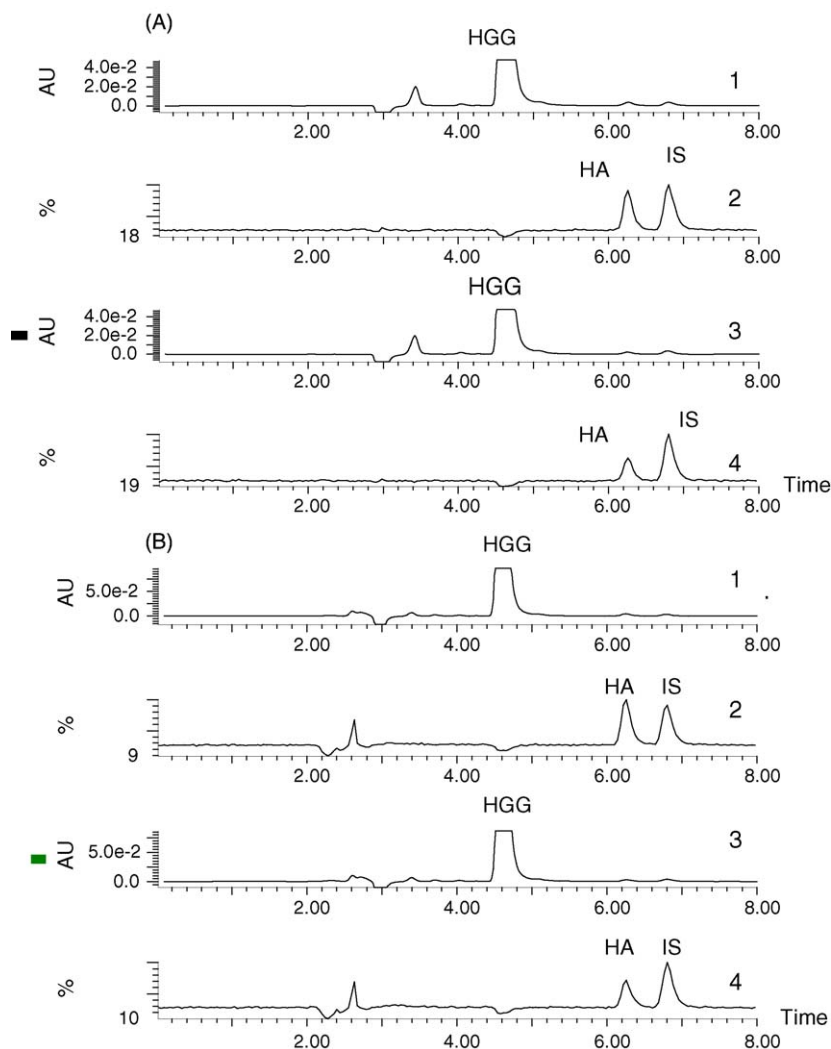


Fig. 3. (A) 0.2 mM HGG hydrolysis catalyzed by purified rabbit ACE (2 μU) for 8 min; (B) 0.2 mM HGG hydrolysis catalyzed by plasma ACE for 16 min. (1) HPLC chromatogram of reaction mixture in control group monitored by UV–vis at 228 nm; (2) corresponding ESI-MS chromatogram of (1) recorded in selected ion monitoring mode; (3) HPLC chromatogram of reaction mixture in the presence of 0.8 ng/ml benazeprilat monitored by UV–vis at 228 nm; (4) corresponding ESI-MS chromatogram of (3) recorded in selected ion monitoring mode.

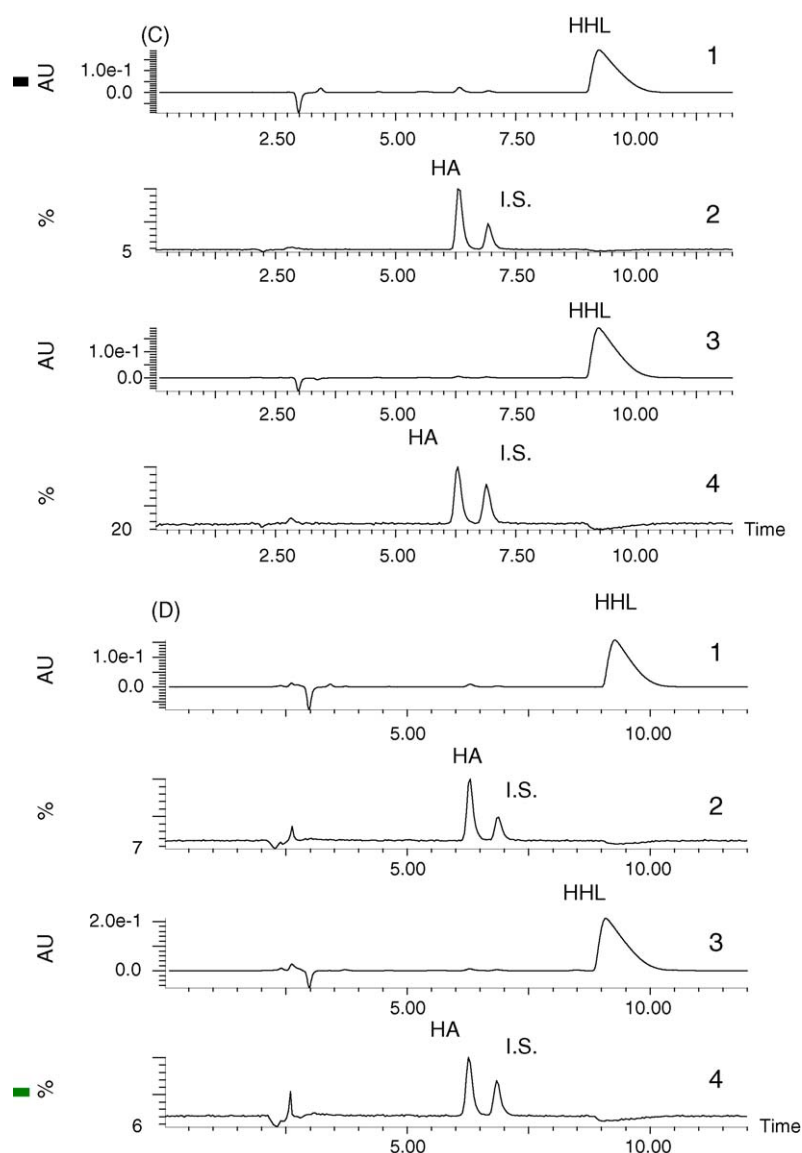


Fig. 4. (C) 0.2 mM HHL hydrolysis catalyzed by purified rabbit ACE (2 mU) for 8 min; (D) 0.2 mM HHL hydrolysis catalyzed by plasma ACE for 16 min. (1) HPLC chromatogram of reaction mixture in control group monitored by UV–vis at 228 nm; (2) corresponding ESI-MS chromatogram of (1) recorded in selected ion mode; (3) HPLC chromatogram of reaction mixture in the presence of 0.8 ng/ml benazeprilat monitored by UV–vis at 228 nm; (4) corresponding ESI-MS chromatography of (3) recorded in selected ion mode.

from purified ACE are relatively smaller than those derived from human plasma ACE, which may be due to the interferences of substrate hydrolysis catalyzed by other carboxypeptidases from plasma [38]. However, there are no significant differences between HHL and HGG hydrolysis catalyzed by the same source of ACE as for average standard deviation. Overall, these results demonstrate an acceptable repeatability, although the standard deviation derived from human plasma ACE might be higher than anticipated.

In order to validate the newly developed method in this paper, IC_{50} (the concentration of inhibitor required to inhibit 50% of the ACE activity) values of benazeprilat obtained by the four assays were compared with those obtained by conventional HPLC method [15,36]. To make sure that the results obtained by the conventional HPLC method were comparable with those

by the proposed method, some modifications were made to the conventional HPLC method, i.e. the concentration of incubation components in both methods were exactly the same except that the incubation time of the HPLC method was prolonged to 40 min for purified lung ACE and 60 min for human plasma ACE. The experiment results for each assay are shown in Table 4. As we can see from Table 3, IC_{50} values obtained by the both methods are in agreement with each other, and the precision (R.S.D.) of the proposed method does not differ greatly with that of the conventional HPLC method. The data obtained by both methods are also in accordance with that reported in the literature ($IC_{50} = 0.79$ ng/ml) [39], which indicates the reliability of this proposed method.

For comparison, the values of kinetic constant K_m for HHL or HGG were also determined from Lineweaver–Burk plots [40] of

Table 4
Comparison of IC₅₀ of benazeprilat obtained by proposed and conventional methods

Assays	IC ₅₀ values (ng/ml) (<i>n</i> = 3)			
	Proposed method		Conventional method	
	Mean	R.S.D.% ^a	Mean	R.S.D.%
HHL and plasma ACE	0.71	7.2	0.78	10.4
HHL and purified ACE	0.65	3.8	0.61	3.4
HGG and plasma ACE	0.58	8.1	0.64	9.2
HGG and purified ACE	0.62	2.9	0.67	4.7

^a R.S.D.%, relative standard deviation percentage.

Table 5
Comparison of *K_m* values obtained by the proposed method with those reported in the literature

Assays	<i>K_m</i> values calculated ^a	<i>K_m</i> values in literature
HHL and purified ACE	2.226 ± 0.29	1.54–2.6
HGG and purified ACE	3.258 ± 0.41	2.6–3.0

^a *n* = 3, mean ± standard deviation.

the reciprocal of reaction velocity versus reciprocal of substrate concentration by both the proposed and the conventional HPLC method (Table 5). Each value was determined from five experiment points with a substrate concentration range of 0.4–4.0 mM. The *K_m* values for HHL and HGG obtained by the both methods were in reasonable agreement with each other, and these values were also in the same range of those reported in the literature [9,15,34].

4. Conclusion

A HPLC-MS method for determination of the ACE inhibitory activity has been developed using HHL or HGG as the substrate, and purified rabbit ACE or human plasma ACE as the source of ACE. This new method turns out to be sensitive, accurate, and reproducible. The small total reaction volume, the low concentration of substrate, and the simple treating procedures are the advantages of the new method. In the conventional HPLC or spectrophotometry method, an incubation period of 30–80 min has been required. However, the total time of the whole procedure for one sample with the novel method described herein is 20 min and 36 min for purified rabbit ACE and blank human plasma, respectively, while the accuracy and the precision of the new method are almost the same as the conventional HPLC method.

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