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Inhibition study of angiotensin converting enzyme by capillary electrophoresis after enzymatic reaction at capillary inlet

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Abstract

Capillary electrophoresis was used to study the inhibition of angiotensin-converting enzyme (ACE) by different inhibitors. Reaction occurred at the capillary inlet during a predetermined waiting period, followed by the electrophoretic separation of the reaction compounds. ACE activity was determined by the quantification of the reaction product, hippuric acid, at 230 nm. The technique was used to study the potency of five different inhibitors (captopril, lisinopril, perindoprilat, quinaprilat and benazeprilat). During a kinetic study, the K_i value of captopril was estimated to be 55.4±8.8 n*M*, a value consistent with previously reported values.

Keywords: Kinetic studies; At-inlet technique; Angiotensin-converting enzyme; Enzymes; Enzyme inhibitors; Hippuric acid

1. Introduction

Angiotensin-converting enzyme (ACE, EC 3.4.15.1) is a relatively non-selective dipeptidyl carboxypeptidase that accepts various substrates, including angiotensin I and bradykinin. ACE plays an important role in the regulation of blood pressure by converting angiotensin I into the vasoconstrictor angiotensin II and also by inactivating the vasodilator bradykinin. The enzyme is found principally on the luminal surface of endothelial cells in contact with the blood, but it is also expressed at lower levels in other cell types [1].

An interest exists in ACE both as a drug target and regarding its role in drug metabolism interactions. Early mechanism studies on angiotensin I conversion and bradykinin hydrolysis, led to the isolation (from snake venom) and the synthesis of small peptide inhibitors. Based on this work and on the modeling studies of carboxy-peptidase active sites, orally active small-molecule inhibitors were then synthesized, such as captopril and lisinopril [1]. These compounds proved to be highly successful in the treatment of hypertension and related target-organ damage, including heart failure and renal insufficiency. Up to now, 17 ACE inhibitors have been developed for clinical use; they are structurally heterogeneous compounds with different pharmacokinetic properties [1]. Furthermore, the serum ACE activity level is a well-established marker for the diagnosis of pathologies such as sarcoidosis, in which increased serum ACE activity has been reported [2].

Due to its importance, many methods have been developed for the determination of ACE activity,

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such as spectrophotometric assays, radioisotopic and fluorimetric methods [3–5].

In spectrophotometric methods for determination of ACE activity, the synthetic tripeptide substrate, hippuryl-L-histidyl-L-leucine (HHL), has been used most widely [2]. ACE releases L-histidyl–L-leucine (HL) and hippuric acid (HA) from this substrate, the latter one can be easily detected by UV at 228 nm:

$$\text{hippuryl-histidyl-leucine} \xrightarrow{\text{ACE}} \text{histidyl-leucine} + \text{hippuric acid} \quad (1)$$

HPLC-based [6,7] and CE-based [8,9] assays were introduced, since the hippuric acid released by the enzymatic reaction cannot be completely separated from the substrate by solvent extraction.

Most of these methods have drawbacks such as the consumption of a relatively high amount of enzyme and the lack of automation. To overcome these limitations, a new CE based method has been developed, in which enzyme and substrate react at the capillary inlet part prior to the electrophoretic separation of the reaction compounds [10]. ACE activity towards the tripeptide substrate HHL was determined by CE with spectrophotometric quantification of the reaction product, hippuric acid, at 230 nm. By using this miniaturized method, a Michaelis–Menten constant was determined for the ACE-assisted cleavage of HHL.

CE is a powerful and relatively new analytical tool, characterized mainly by high resolution separations, short analysis times and low sample load. In general, separations applied to biochemical systems are well suited to miniaturization because samples typically have low volumes.

In this work, we performed a kinetic study of the inhibitor captopril and we investigated the relative inhibitory potency of five different ACE inhibitors (i.e. captopril, lisinopril, perindoprilat, quinaprilat and benazeprilat) by means of the previously developed in-capillary system.

2. Materials and methods

2.1. CE instrumentation

All experiments were carried out on a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA, USA). On-line detection was performed at 230 nm with a diode array detector. Data collection and peak area analysis were performed by 32 Karat software (version 5.0). Calculation of inhibition constants was done by means of SigmaPlot 2001 software (version 7.101). Uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 31.2 cm (21 cm from the injection side to the detector)×75 μm I.D. were used. The capillary was thermostated by liquid cooling at 37 °C unless specified otherwise.

2.2. Materials and reagents

ACE from rabbit lung, HHL and captopril were purchased from Sigma–Aldrich (Steinheim, Germany). Lisinopril dihydrate was donated by Merck Sharp and Dohme (Brussels, Belgium) and AstraZeneca (Brussels, Belgium). Perindoprilat was synthesized by Servier (Paris, France). Quinaprilat was a gift from Pfizer (Brussels, Belgium) and benazeprilat was donated by Novartis (Basel, Switzerland). Solutions of ACE, HHL and the inhibitors (captopril, lisinopril, perindoprilat, benazeprilat and quinaprilat) were prepared in a 10-m*M* HEPES buffer {2-[4-(2-hydroxyethyl)-1-piperazine]ethane sulfonic acid, Sigma–Aldrich} adjusted with 1 *M* NaOH (BDH Laboratory Supplies, Poole, UK) to pH 8.0 at 37 °C, that contained 150 m*M* NaCl (BDH). All solutions were prepared with Milli-Q water (Millipore, Milford, MA, USA) and filtered through 0.2-μm nylon filters (Alltech, Lokeren, Belgium).

2.3. At-inlet reaction

The CE run buffer consisted of 150 m*M* HEPES adjusted with 1 *M* NaOH to pH 8.0 at 37 °C. Before use, a new capillary was treated with 0.1 *M* NaOH for 2 h. Prior to analysis, the capillary was conditioned by a wash cycle at 20 p.s.i. starting with 0.1 *M* NaOH for 5 min, followed by a 2-min rinse with Milli-Q water and a 10 min rinse with run buffer (1 p.s.i. = 6894.76 Pa). After each analysis of reaction mixture the capillary was rinsed with 0.1 *M* NaOH, water and run buffer for 1, 1 and 3 min, respectively.

The enzyme solution and the substrate solution, with or without inhibitor, were introduced into the

inlet part of the capillary by a sandwich injection mode, i.e. enzyme solution (0.3 p.s.i., 5 s)–substrate solution (0.3 p.s.i., 5 s)–enzyme solution (0.3 p.s.i., 5 s). After each injection step of substrate or enzyme, the capillary ends (and electrodes) were dipped into water in order to prevent sample carry over. Before and after the sandwich injection mode a small plug of water was injected hydrodynamically (0.1 p.s.i., 5 s). The consecutively injected plugs were allowed to stand during a predetermined waiting period and then a voltage of 6 kV was applied. The mixing of substrate and a specific inhibitor was performed immediately before the measurements to prevent any reaction. ACE activity (or the extent of reaction) was determined by the corrected peak area, i.e. the peak area divided by the migration time, of the product HA. As peak areas have no physical units, the reaction velocities are scaled arbitrarily.

3. Results and discussion

3.1. Enzyme inhibition by CE

Capillary electrophoretic systems have been successfully applied for in-line enzymatic reactions by a methodology known as electrophoretically mediated microanalysis (EMMA), firstly described by Bao and Regnier [11]. EMMA utilizes the different electrophoretic mobilities of enzyme and substrate to initiate reaction inside the capillary, which is used as the reaction vessel. Subsequently, the different reaction compounds are transported to the detector by electrophoresis, where they are individually detected. EMMA methodology has been applied in a number of biochemical systems, including assays of enzyme activity [12–14], determination of Michaelis constants [15–18] and inhibition constants of enzyme inhibitors [19–21].

A related, but different approach has been adopted for the kinetic study of ACE activity towards the peptide substrate HHL [10]. The compounds were introduced successively to the capillary inlet part by hydrodynamic injection steps and the injected plugs were then allowed to stand for an adequate period of time. The compounds reacted inside the capillary, while no voltage was applied. Immediately after the reaction, the compounds were separated by the application of the voltage and analyzed. Unlike in EMMA analysis, the compounds were not electrophoretically mixed prior to reaction in this method.

An identical setup was used in this study, the only modification was the addition of the inhibitors to the substrate solution. Fig. 1 provides a schematic overview of the different injection steps of the at-inlet reaction. In a first step (1) a plug of water (W) is injected on the capillary. Subsequently, a plug of enzyme (E) solution, a plug of substrate (S) solution (with or without inhibitor) and a plug of enzyme (E) solution are injected hydrodynamically on the capillary (2). Finally, a plug of water (W) is injected on the capillary (3). The plugs are then allowed to react while the capillary end is dipped into the run buffer. After reaction, a voltage of 6 kV is applied and the reaction compounds are swept towards the detector end of the capillary.

A typical electropherogram of the enzymatic reaction is shown in Fig. 2A, in which an ACE solution of 0.42 U/ml and a HHL (substrate) concentration of 4.92 m*M* were used. This substrate concentration is about 4.5 times the K_M (Michaelis–Menten constant), higher concentrations cause substrate inhibition. The inhibitory activity of captopril on the ACE induced cleavage of HHL can be seen from the electropherogram measured under identical

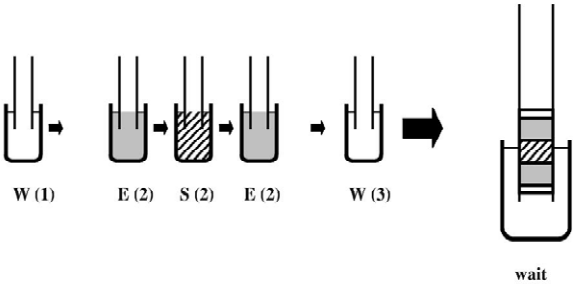

Fig. 1. Schematic illustration of the introduction of the different plugs to the inlet of a capillary: a plug of water is introduced at 0.1 p.s.i. (5 s) before (1) and after (3) the sandwich injection of enzyme and substrate; (2) sandwich injection of enzyme and substrate (3×0.3 p.s.i. during 5 s); the consecutively injected plugs are then allowed to stand during a predetermined waiting period while the capillary end is dipped in run buffer; subsequently voltage is applied and the reaction compounds are transported to the detector. □ water (W); ▭ enzyme solution (E); ▨ substrate solution with our without inhibitor (S).

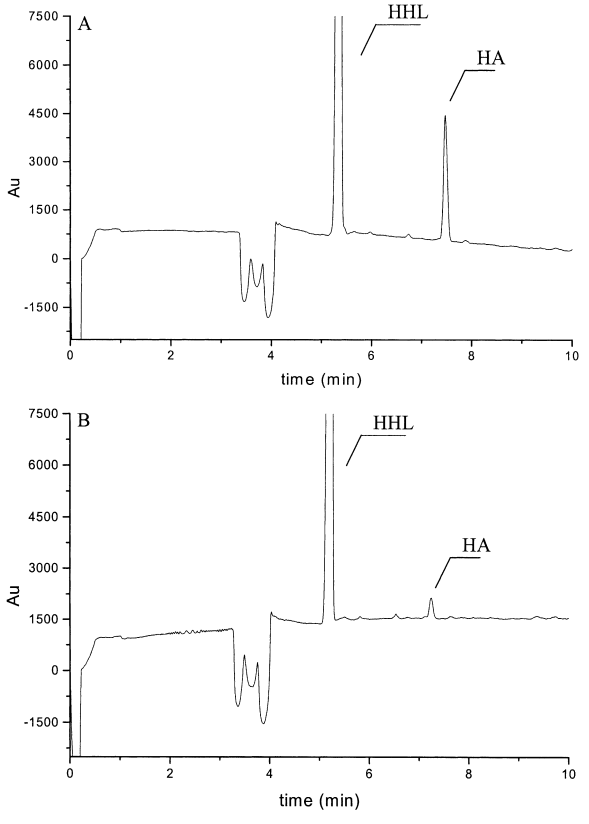

Fig. 2. Typical electropherogram obtained after on-line reaction at the capillary inlet, without (A) and with (B) the inhibitor captopril added to the substrate plug. The concentration of ACE: 0.42 U/ml, HHL: 4.92 m*M* and captopril: 800 n*M*. Waiting period of 0 min. CE conditions: run buffer: 150 m*M* HEPES (pH 8.0); applied voltage: 6 kV; current: 76 μA; detection at 230 nm, capillary cartridge temperature: 37 °C.

conditions, but with the addition of 800 n*M* of captopril to the substrate solution (Fig. 2B).

3.2. Apparent mobility of the ACE inhibitors

Since the velocity of the enzymatic reaction is measured by the peak area of the formed HA, it is important that no peaks are located underneath the product peak at 230 nm. The inhibitors are acids, as well as the reaction product HA. The apparent mobility of HA was determined to be $2.162 \cdot 10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$ in the 150 m*M* HEPES (pH 8.0; 37 °C) background electrolyte. As shown in Table 1, all the inhibitors had different apparent mobilities and migrated faster than the product HA. The apparent mobility was calculated from the migration time of the compound, taking into account the capillary length and the applied voltage.

Table 1
Apparent mobilities and percent of inhibition for the different inhibitors

Inhibitor	Apparent mobility ($cm^2\ V^{-1}\ s^{-1}$)	Percent of inhibition (%)
Perindoprilat	$2.389 \cdot 10^{-4}$	44.2
Lisinopril	$3.390 \cdot 10^{-4}$	42.6
Benazeprilat	$2.287 \cdot 10^{-4}$	42.5
Quinaprilat	$2.391 \cdot 10^{-4}$	41.0
Captopril	$2.473 \cdot 10^{-4}$	35.9

Mobility determined at a concentration of 1 mg/ml.
Inhibition (%) is the average value for three determinations at a $5 \cdot 10^{-8}$ *M* concentration.

3.3. Stability of captopril

In aqueous solution captopril undergoes an oxygen facilitated oxidation at its thiol group to yield captopril disulfide. The degradation of captopril could be followed in our system, since the inhibitory activity of the compound decreased over time which led to an increase in product formation. Fig. 3 shows the amount of HA determined after in-capillary reaction in the presence of 50 n*M* of captopril, measured at time intervals of 24 h. As can be seen in Fig. 3, the amount of HA increased by 73% over the 4-day stability experiment.

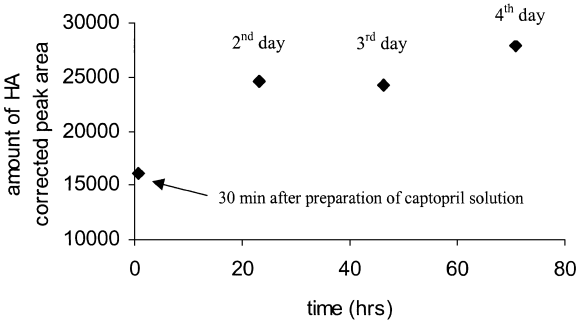

Fig. 3. The amount of HA after at-inlet reaction between ACE (0.42 U/ml) and HHL (5 m*M*) in the presence of captopril (50 n*M*), determined at regular time periods of 24 h after the preparation of the captopril solution. Waiting period of 1 min. CE conditions: see Fig. 2.

Captopril is optimally stable below pH 3.5, but this is not an ideal pH for the ACE enzymatic activity. The addition of water-soluble antioxidants can affect the oxidation mechanism, but we chose not to incorporate extra compounds in the reaction mixture except for the inhibitors. Therefore, captopril solutions were freshly prepared and analysis was always done 30 min after the preparation.

3.4. Inhibition curve of captopril

To verify that the developed method can generate comparable results to those obtained with other methods, an enzyme inhibition study was performed with the inhibitor captopril. Six different captopril concentrations were tested: 0, 1.26, 5.07, 25.3, 792 n*M* and 39.6 μ*M* and the amount of HA was determined after in-capillary reaction. The percentage of inhibition was determined according to the following equation:

$$\% = 100 - \left(\frac{x}{\text{blank}} \cdot 100\right) \quad (2)$$

where x represents the amount of HA determined at a given concentration of captopril and the blank or negative control value is the amount of HA determined without captopril being present (concentration of 0 n*M*). In Fig. 4, the percentage of inhibition (right *y*-axis: solid line) is expressed against the captopril concentration (μ*M*). The product curve (left *y*-axis: dotted line) represents the amount of product determined after in-capillary reaction between ACE (0.42 U/ml) and HHL (2.50 m*M*) in the presence of captopril. At the highest captopril concentration of 39.6 μ*M*, a 92.6% inhibition was obtained. This means that complete inhibition was not reached, even at high captopril concentrations. A possible explanation is the fact that the enzyme was not preincubated with the inhibitor, prior to the injection on the capillary and the start of the reaction. Another reason might be the dilution factor, which originates from the mixing process of the plugs during the at-inlet reaction [10]. The measured IC_{50} (concentration of compound at which the reaction was inhibited by 50%) with the in-capillary assay was approximately 0.33 μ*M*. This

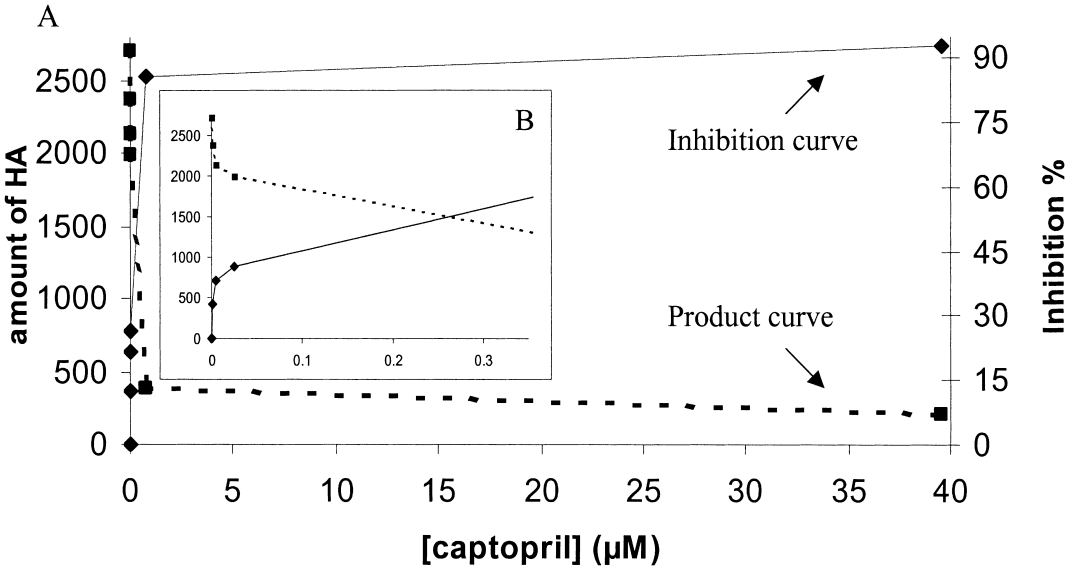

Fig. 4. (A) Left *y*-axis, product curve (dotted line), the amount of HA determined after at-inlet reaction between ACE (0.42 U/ml) and HHL (2.50 m*M*), when the captopril concentration was varied between 0 and 39.6 μ*M*. The extent of the reaction was determined by the corrected peak area of HA. Waiting period of 0 min. The right *y*-axis (solid line) represents the percentage of inhibition (%) for each captopril concentration. CE conditions: see Fig. 2. The insert (B) shows an enlarged view of the lower concentration range.

determined value is similar to literature values where IC_{50} values for captopril range from 2.27 n*M* to 0.58 μ*M* [22–24].

3.5. K_i determination of captopril

We assumed a competitive mechanism for the inhibitor captopril [23] in order to estimate the enzyme inhibition constant or the K_i value of this compound. The Michaelis–Menten plots for the enzymatic cleavage of HHL at different concentrations of the inhibitor captopril (0, 50, and 100 n*M*) are given in Fig. 5A. Each concentration point was analyzed in triplicate. From these plots, the Lineweaver–Burk plots (double reciprocal plots) were constructed (shown in Fig. 5B). From these data, a K_i value for captopril of 55.4±8.8 n*M* could be computed. Depending on the nature of the assay and on the origin of the enzyme, reported values range from 0.33 to 72 n*M* [23,25,26], which means that the described method can estimate the K_i value of a given inhibitor.

Furthermore, this K_i value corresponds to the calculated value of 62.1 n*M*, derived from the IC_{50} value of the inhibition curve (see Section 3.4), calculated by Cheng and Prusoff's equation [27] giving the relationship between K_i and the concentration of inhibitor for 50% inhibition (IC_{50}):

$$IC_{50} = K_i(1 + [S]/K_M) \quad (3)$$

The K_M value of 1.16 m*M* for ACE with HHL as a substrate was determined previously [10] for three different enzyme concentrations (0.42, 0.33 and 0.25 U/ml), while a HHL concentration of 5.0 m*M* was used.

When the different concentrations of captopril were investigated, unpredictable migration time shifts and even current breakdown were sometimes seen. We do not have a straightforward explanation: the reactive thiol group of the inhibitor might react with the capillary wall or with other compounds of the reaction mixture.

3.6. Comparison of different inhibitors

To determine the inhibitory potency of the different ACE inhibitors with the developed at-inlet method, we compared the effect of captopril, lisinopril, quinaprilat, benazeprilat and perindoprilat, all at a concentration of $5 \cdot 10^{-8}$ *M*. None of the inhibitors were preincubated with the enzyme; the reaction was initiated by the consecutive injection steps of the enzyme and substrate solution on the capillary. The ACE concentration used was 0.42 U/ml and HHL was used at 5.00 m*M*. Table 1 shows the percentage of inhibition at a $5 \cdot 10^{-8}$ *M* concentration of inhibitor on the ACE induced cleavage of HHL, compared to a blank solution (substrate without inhibitor). Each analysis was performed in triplicate. Captopril showed to be less potent (36% inhibition at a concentration of $5 \cdot 10^{-8}$ *M*) than the other inhibitors, while perindoprilat (44% inhibition) showed to be the most potent inhibitor. These findings are in agreement with literature, where perindoprilat is known to be a very potent inhibitor (more potent than lisinopril and captopril) [28] and lisinopril is known to be more potent than captopril [30]. In other studies, however, quinaprilat is more potent than benazeprilat and lisinopril [29] and this was not found in our experiments, although the difference in inhibition at $5 \cdot 10^{-8}$ *M* was not very high.

Nevertheless, the difference in inhibitory potency of the five inhibitors determined after in-capillary reaction, implies that this method can be used for preliminary ACE inhibitor screening.

4. Conclusion

The inhibition of angiotensin-converting enzyme with different inhibitors was studied by means of CE. Reaction occurred at the capillary inlet between the enzyme and its substrate HHL in the presence of an ACE inhibitor. ACE activity was determined spectrophotometrically by the quantitation of the product HA after an electrophoretic separation of the reaction compounds. The method was not only used to compare inhibitory potencies of different inhibitors but also to estimate the K_i constant of captopril. The K_i of 55.4 n*M* determined for this inhibitor is in agreement with literature values. Since the capillary is used as a micro vessel in this technique, all the necessary steps (reaction, separation and quantitation) are combined in one fully automated and miniaturized assay. The obtained results can contribute to further applications of this

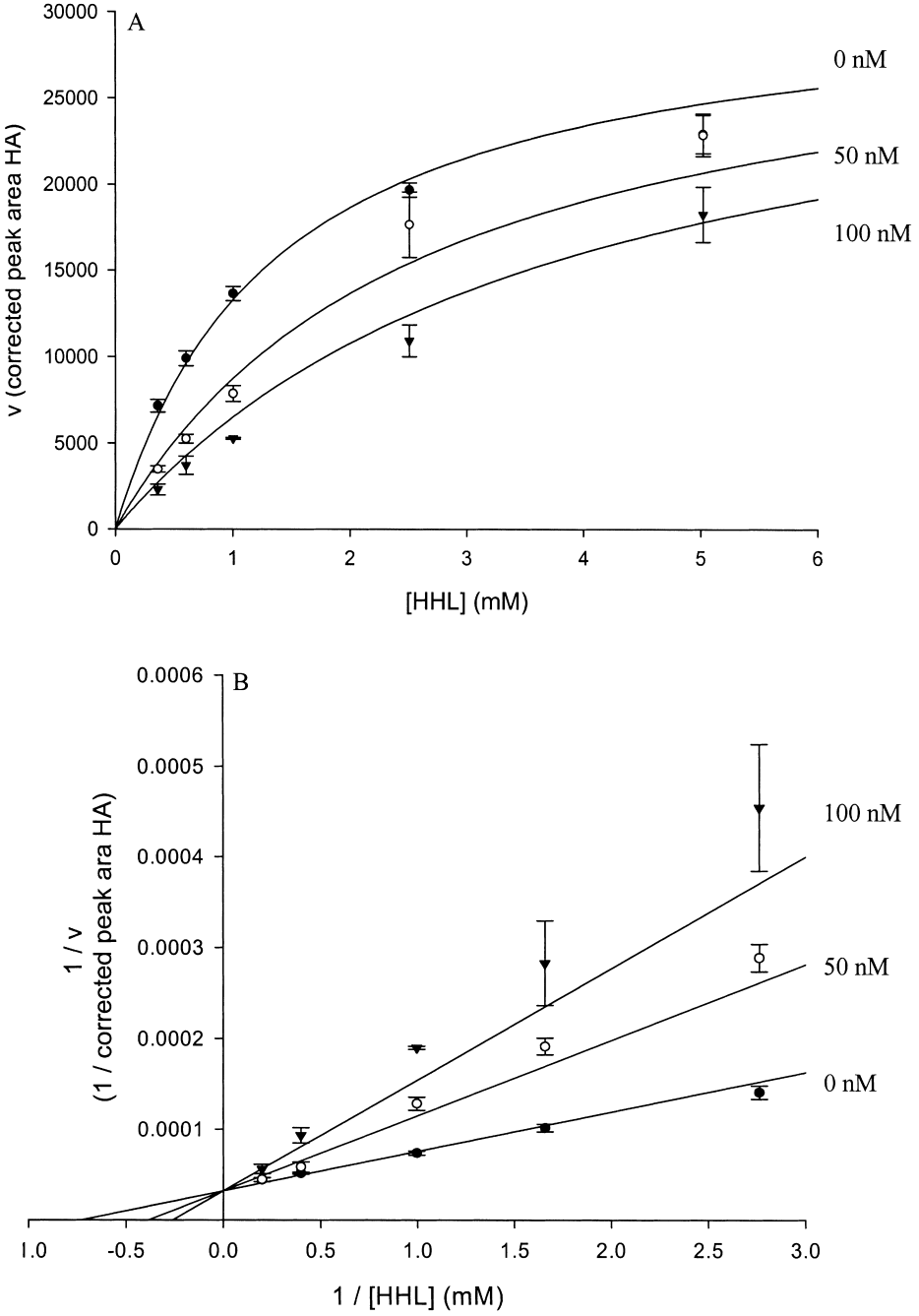

Fig. 5. (A) The Michaelis–Menten plots for the enzymatic reaction of ACE inhibited by (●) 0 nM; (○) 50 nM; and (▼) 100 n*M* captopril. The HHL concentration was varied between 0.361 and 5.02 m*M*. Each concentration point was analysed in triplicate. In-capillary reaction at capillary inlet, waiting period of 0 min. CE conditions: see Fig. 2. (B) The corresponding Lineweaver–Burk plots of ACE inhibition by captopril: (●) 0 nM; (○) 50 nM; and (▼) 100 nM.

approach as in the areas of drug development and inhibitor screening.

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References

[1] M.A. Zaman, S. Oparil, D.A. Calhoun, Nat. Rev. Drug Discov. 1 (2002) 621.
[2] B. Bénéteau-Burnat, B. Baudin, Crit. Rev. Clin. Lab. Sci. 28 (1991) 337.
[3] D.W. Cushman, H.S. Cheung, Biochem. Pharmacol. 20 (1971) 1637.
[4] J.W. Ryan, A. Chung, C. Ammons, M.L. Carlton, Biochem. J. 167 (1977) 501.
[5] J. Friedland, E. Silverstein, Am. J. Clin. Pathol. 66 (1976) 416.
[6] S.G. Chiknas, Clin. Chem. 25 (1979) 1259.
[7] Q.C. Meng, E. Balcells, L. Dell'italia, J. Durand, S. Oparil, Biochem. Pharmacol. 50 (1995) 1445.
[8] Z.K. Shihabi, J. Chromatogr. A 853 (1999) 185.
[9] R.-Z. Zang, X.-H. Xu, T.-B. Chen, L. Li, P.-F. Rao, Anal. Biochem. 280 (2000) 286.
[10] S. Van Dyck, S. Vissers, A. Van Schepdael, J. Hoogmartens, J. Chromatogr. A 986 (2003) 303.
[11] J. Bao, F.E. Regnier, J. Chromatogr. 608 (1992) 217.
[12] K.J. Miller, I.K. Leesong, J.M. Bao, F.E. Regnier, F.E. Lytle, Anal. Chem. 65 (1993) 3267.
[13] D. Wu, F.E. Regnier, M.C. Linhares, J. Chromatogr. B 657 (1994) 357.
[14] T. Watanabe, A. Yamamoto, S. Nagai, S. Terabe, Electrophoresis 19 (1998) 2331.
[15] J. Saevels, A. Van Schepdael, J. Hoogmartens, Electrophoresis 17 (1996) 1222.
[16] S. Van Dyck, A. Van Schepdael, J. Hoogmartens, Electrophoresis 22 (2001) 1436.
[17] S. Nováková, Z. Glatz, Electrophoresis 23 (2002) 1063.
[18] S. Van Dyck, A. Van Schepdael, J. Hoogmartens, Electrophoresis 23 (2002) 2854.
[19] J. Saevels, K. Van den Steen, A. Van Schepdael, J. Hoogmartens, J. Chromatogr. A 745 (1996) 293.
[20] A.R. Whisnant, S.E. Johnston, S.D. Gilman, Electrophoresis 21 (2000) 1341.
[21] S. Nováková, M. Telnárová, Z. Glatz, J. Chromatogr. A 990 (2003) 189.
[22] R.D. Carr, L. Higgs, P.G. Killingback, A.K. Nicol, S.E. O'Connor, A. Robson, E. Wells, W.T. Simpson, Br. J. Pharmacol. 100 (1990) 83.
[23] E.G.P. Udupa, N.M. Rao, Biochem. Mol. Biol. Int. 43 (1997) 1063.
[24] Y. Inada, Z. Terashita, Y. Imura, M. Tanabe, K. Nishikawa, S. Kikuchi, Jpn. J. Pharmacol. 42 (1986) 99.
[25] R. Shapiro, J.F. Riordan, Biochemistry 23 (1984) 5225.
[26] R.G. Almquist, W.R. Chao, M.E. Ellis, H.L. Johnson, J. Med. Chem. 23 (1980) 1392.
[27] Y.Ch. Cheng, W.H. Prusoff, Biochem. Pharmacol. 22 (1973) 3099.
[28] M. Barthelmebs, M. Devissaguet, J.L. Imbs, Clin. Exp. Theor. Pract. A 11 (1989) 471.
[29] D.W. Cushman, F.L. Wang, W.C. Fung, G.J. Grover, C.M. Harvey, R.J. Scalese, S.L. Mitch, J.M. Deforrest, Br. J. Clin. Pharmacol. 28 (1989) 115S.
[30] C.I. Johnston, B. Fabris, H. Yamada, F.A.O. Mendelsohn, R. Cubela, D. Sivell, B. Jackson, J. Hypertens. 7 (1989) S11.