Available online at www.sciencedirect.com
SCIENCE DIRECT®

Journal of Chromatography A, 986 (2003) 303–311

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Kinetic study of angiotensin converting enzyme activity by capillary electrophoresis after in-line reaction at the capillary inlet

Sigrid Van Dyck, Stijn Vissers, Ann Van Schepdael*, Jos Hoogmartens

Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen, Katholieke Universiteit Leuven, Van Evenstraat 4, B-3000 Leuven, Belgium

Received 22 August 2002; received in revised form 18 November 2002; accepted 2 December 2002

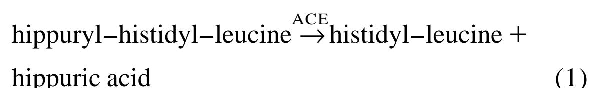
Abstract

The in-capillary reaction of angiotensin converting enzyme (ACE) with the tripeptide substrate hippuryl–L-histidyl–L-leucine was studied. ACE activity was determined by the quantitation of the product, hippuric acid, at 230 nm. Reaction occurred at the capillary inlet during a predetermined waiting period, followed by the electrophoretic separation of the compounds. When the set-up was reversed, i.e. reaction at the opposite side after short-end injection of enzyme and substrate, separation was achieved in less than 5 min. Using the Lineweaver–Burk equation, an average Michaelis constant for ACE from rabbit lung was calculated to be 1.16±0.12 m*M*, a value consistent with previously reported data.
© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Kinetic study; Michaelis–Menten analysis; At-inlet reaction; Angiotensin-converting enzyme; Enzyme; Peptides; Hippuric acid

1. Introduction

Angiotensin converting enzyme (ACE, EC 3.4.15.1) is a chloride-dependent peptidase that catalyzes the release of a C-terminal dipeptide from its substrate molecules [1]. In spectrophotometric methods for determination of ACE activity, the synthetic tripeptide, 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 can be easily detected by UV detection at 228 nm:



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

ACE plays an important role in the regulation of blood pressure by converting the peptide angiotensin I into the vasoconstrictor angiotensin II and also by inactivating the vasodilator bradykinin [2].

Increased serum ACE activity has been reported in pathologies such as sarcoidosis [3], where the serum ACE activity level is a well-established marker for the diagnosis [2]. Several synthetic ACE inhibitors (captopril, ramipril, lisinopril, etc.) are used in the treatment of hypertension and congestive heart failure [4,5].

Clearly, determination of ACE activity is important clinically as well as in ACE inhibitor screening.

*Corresponding author. Tel.: +32-16-32-3443; fax: +32-16-32-3448.
E-mail address: ann.vanschepdael@farm.kuleuven.ac.be (A. Van Schepdael).

0021-9673/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0021-9673(02)01995-7

Many assay methods have been developed such as spectrophotometric assays [6,7], radioisotopic [8,9] and fluorimetric methods [10]. HPLC-based assays were introduced, since the hippuric acid released by the enzymatic reaction cannot be completely separated from the substrate by solvent extraction [11–13]. More recently, assays based on capillary electrophoresis (CE) have been published [14,15]. In the reported CE-based assays, CE is used for the separation of the compounds of the reaction mixture, after off-line incubation.

CE is a powerful and relatively new analytical tool, characterized by high resolution separations, short analysis times and low sample load. A characteristic property of CE is the fact that it can be performed in free solutions. This implies that the capillary, the separation tool, can also be used as a small reaction vessel. In this manner, all the different assay steps (i.e. reaction, separation, quantitation) can be combined in one automated, microscale assay.

CE systems have been successfully applied for in-line enzymatic reactions by a methodology known as electrophoretically mediated microanalysis (EMMA) [16]. EMMA utilizes the different electrophoretic mobilities of enzyme, substrate and product to initiate reaction inside the capillary and to separate the components from each other for final on-line quantitation. This technology has been used for different biochemical systems: enzyme activity assays [17–20], kinetic studies with the determination of Michaelis constants [21–23] or the study of inhibitors and inhibition constants [24,25], etc.

In order to develop an in-capillary assay for ACE, we obtained the best results when we applied the at-inlet reaction technique on this enzymatic system. After a sandwich mode injection, enzyme–substrate–enzyme, the overlayed plugs of enzyme and substrate were allowed to react at the capillary inlet. Subsequently voltage was applied and the reaction compounds were separated and quantified by CE. Unlike in a typical EMMA analysis, enzyme and substrate were not electrophoretically mixed prior to reaction.

The at-inlet technique has been described by Taga et al. [26], who performed CE with derivatization reactions at the capillary inlet using amino acids as model compounds.

2. Materials and methods

2.1. CE instrumentation

All experiments were carried out on a P/ACE MDQ CE system (Beckman Instruments, Fullerton, CA, USA). On-line detection was performed at 230 nm with a diode array detection system. Data collection and peak area analysis were performed by P/ACE MDQ software (version 1.5). 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. CE conditions

The CE run buffer consisted of 150 m*M* HEPES {2-[4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid, Sigma–Aldrich, Steinheim, Germany} adjusted with 1 *M* NaOH (BDH, Poole, UK) to pH 8.0 at 37 °C. Before use, a new capillary was treated with 0.1 *M* NaOH for 2 h. At the beginning of each day, the capillary was conditioned by a wash cycle at 20 p.s.i. (1 p.s.i. = 6894.76 Pa) starting with 0.1 *M* sodium hydroxide for 5 min, followed by a 2-min rinse with Milli-Q water (Millipore, Milford, MA, USA) and a 10 min rinse with run buffer. 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. All solutions were prepared with Milli-Q water and filtered through 0.2 μm nylon filters (Alltech, Lokeren, Belgium).

After each injection step of substrate or enzyme, the capillary ends (and electrodes) were dipped into water in order to prevent sample carry over.

HL was purchased from Bachem (Bubendorf, Switzerland) and HA was purchased from Sigma–Aldrich. HL and HA were dissolved in a 10 m*M* HEPES buffer (pH 8.0) which also contained 150 m*M* NaCl.

2.3. At-inlet reaction (after long-end injection)

ACE from rabbit lung and HHL were purchased from Sigma–Aldrich. Solutions of ACE and HHL

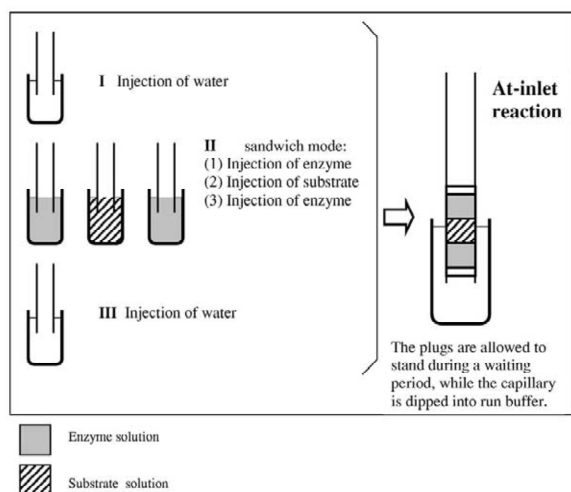


Fig. 1. Schematic illustration of the different steps of the at-inlet reaction: (I) and (III) a plug of water is introduced before and after the sandwich injection of enzyme and substrate in the capillary by a hydrodynamic injection (0.1 p.s.i., 5 s); (II) enzyme and substrate are injected hydrodynamically on the capillary by the sandwich injection mode: (1) injection of enzyme (0.3 p.s.i., 5 s); (2) injection of substrate (0.3 p.s.i., 5 s); (3) injection of enzyme (0.3 p.s.i., 5 s); the overlayed plugs are then allowed to stand during a predetermined waiting period; subsequently voltage is applied and the reaction compounds, together with the in-line generated reaction product, are transported to the detector.

were prepared in a 10 m*M* HEPES buffer at pH 8.0, that contained 150 m*M* NaCl (BDH). Solutions of ACE and HHL were stored at 4 °C, before use they were allowed to adjust to room temperature. Fig. 1 provides a schematic overview of the different steps of the at-inlet enzymatic reaction. Enzyme and substrate are introduced into the long-end of the capillary by the 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). Before and after the sandwich injection mode a small plug of water is hydrodynamically injected (0.1 p.s.i., 5 s). The overlayed plugs are allowed to stand during a predetermined waiting period and then voltage is applied at 6 kV, with the anode on the injection side and the cathode on the detector side. The different steps of the method, from wash procedure to injection and run conditions, are provided in Table 1.

2.4. Reaction at the opposite side (after short-end injection)

In this set-up the whole at-inlet reaction procedure was reversed. Plugs of enzyme and substrate were introduced by a sandwich injection at the opposite side of the capillary, the end of the capillary nearest

Table 1
Wash, injection and run procedures of the at-inlet reaction

Wash procedure						
Solution	Pressure (p.s.i.)	Time (s)				
(1) 0.1 *M* NaOH	20	60				
(2) Water	20	60				
(3) Run buffer	20	180				
Injection and run procedure						
Steps	Plugs	Pressure (p.s.i.)	Time (s)	NaCl (m*M*)	HEPES (m*M*)	Voltage (kV)
I	Water	0.1	5	0	0	
II (1)	ACE	0.3	5	150	10	
II (2)	HHL	0.3	5	150	10	
II (3)	ACE	0.3	5	150	10	
III	Water	0.1	5	0	0	
IV	/(waiting time)	/	60	/	/	/
V	Run buffer	/	600	0	150	6

Run buffer consists of 150 m*M* HEPES at pH 8.0 (37 °C).

the detector. After the standing period, a reversed polarity voltage of −6 kV was applied and the compounds were swept to the detection window. Separation takes place between the regular capillary outlet and the detection window.

2.5. Calibration of HA concentration

A standard solution of the product was prepared by dissolving HA in 10 m*M* HEPES buffer at pH 8.0, containing 150 m*M* NaCl. A series of HA solutions was obtained from the standard solution by dilution with the same buffer over a concentration range of 0.030–4.821 m*M*. Injections were performed in triplicate. The calibration curve was obtained by plotting the corrected peak area (peak area divided by migration time) of HA against its concentration.

3. Results and discussion

3.1. Quantitative analysis

The determination of ACE activity is achieved by measuring the corrected peak area of HA formed during the enzymatic reaction. Therefore, a strict linear correlation between HA concentration and the corrected peak area is necessary. In the regression equation ($y = 16274x + 2130$; $S_{y,x} = 1133$), y is the corrected peak area and x represents the HA concentration in m*M*. A correlation coefficient (r) of 0.9994 ($n = 3$) was calculated for a HA concentration range from 0.030 to 4.821 m*M*. The limit of quantitation (LOQ) was found to be 4.5 μ*M* HA (RSD 7.4% for $n = 6$). The limit of detection (LOD) of HA was found to be 1.8 μ*M*. According to our calculations, 37.2 nl was injected into the capillary (0.3 p.s.i., 5 s), which corresponds to an injected amount of 33.8 pg (LOQ) or 14.1 pg HA (LOD).

3.2. Development of in-capillary activity assay

The use of high ionic strength buffers in CE results in the generation of a substantial current inside the capillary and thus loss of peak efficiency due to a Joule-heating effect [27]. Unfortunately, the ACE catalyzed reaction is highly chloride-dependent [6] and therefore desalting of the reaction mixtures prior to CE analysis has been proposed [28]. For the development of an in-line method, electrophoretic conditions are required that are favourable for the separation of the reaction compounds and for the enzymatic reaction itself. In reported assay methods for ACE, concentrations of chloride in the reaction mixture varied between 0.15 and 1.1 *M* [6,7,14]. We therefore dissolved enzyme and substrate in a 10 m*M* HEPES buffer that contained 150 m*M* NaCl. HEPES is an inert buffer for the ACE induced cleavage of HHL [29] and has the advantage of generating only a minor current in the capillary due to its low conductivity. Since it can be used at a high concentration, the background electrolyte consisted of 150 m*M* HEPES buffer (without NaCl), which generated a 76 μA current at 6 kV (capillary cartridge at 37 °C).

In CE, samples are preferably dissolved in water or in a 1:10 dilution of the run buffer in order to concentrate (stack) the sample on the capillary [27]. The injection of a sample with an ionic strength higher than the background electrolyte results in band broadening and a decreased signal-to-noise ratio. Although the injected enzyme and substrate solutions contained a high concentration of NaCl (150 m*M*), no strong band broadening can be seen in the electropherograms (Fig. 2). This can be due to the fact that sodium chloride in a sample can occasionally promote stacking while in other instances it causes band broadening, this effect is referred to as sample self-stacking [30]. Before and after the sandwich injection of enzyme and substrate, a small water plug was injected, because this step improved the electropherograms. In many types of stacking an additional plug of water is introduced before the sample is injected on the capillary [30].

Although somewhat less sensitive, 230 nm was chosen for the enzyme assay, a more common wavelength on most instruments compared to the 228 nm maximum for HA detection. Since the velocity of the enzymatic reaction is measured by the peak area of the formed HA, it is important that there are no peaks located underneath the HA peak. The other product of the reaction, the dipeptide HL, absorbs at 230 nm. The dipeptide comigrated with the substrate and did not interfere with HA quantitation. The small

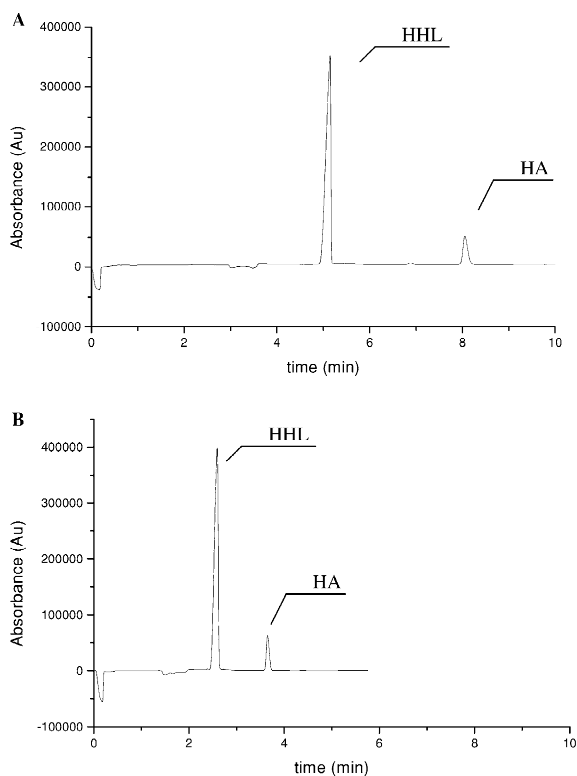


Fig. 2. (A) Electropherogram after in-capillary reaction at the capillary inlet (long-end injection), applied voltage: 6 kV. Concentration of ACE: 0.42 U/ml, HHL: 4.92 m*M*. CE conditions: run buffer: 150 m*M* HEPES (pH 8.0); current: 76 μA; detection at 230 nm, capillary cartridge temperature: 37 °C; waiting period of 1 min. (B) Electropherogram after in-capillary reaction at the opposite side (short-end injection), applied voltage: −6 kV, other conditions identical to (A).

peaks in front of the substrate peak are related to the enzyme solution.

3.3. At-inlet reaction

When we let the plugs of enzyme and substrate mix electrophoretically in the capillary, very high RSD values (>20%) were obtained because the peak area of HA was divergent. A possible explanation would be that the enzymatic reaction is very sensitive to variations in the in-capillary contact period of the enzyme and substrate plugs. This contact period can vary from run to run when the electroosmotic flow changes, when the capillary wall changes due to adsorption phenomena, etc.

However, with the at-inlet technique, the compounds are introduced successively to the capillary inlet part and the injected plugs are allowed to stand for an adequate period of time. The compounds will react inside the capillary, while no voltage is applied. Immediately after the reaction, the compounds are analyzed by applying a voltage (6 kV, normal polarity) and separation is completed within 10 min.

Since UV detection is used, the system lacks concentration sensitivity. When we studied catechol-*O*-methyltransferase activity by EMMA, we were not able to perform a full kinetic analysis due to the limitations of UV detection [31]. The use of another detection mode (e.g. fluorescence detection) would increase the sensitivity, but UV detection is commonly used in CE due to its simplicity and low operational costs. Increase of the waiting period will also enhance the sensitivity by an increase in product area but this will also increase the total analysis time. Solutions can be injected on the capillary in either tandem (substrate–enzyme) or sandwich mode (enzyme–substrate–enzyme). The latter mode provided a 46% increase in product corrected peak area, tested under identical conditions. Therefore, the sandwich injection mode was chosen, although there are drawbacks. In in-line enzymatic assays, a small variation in the injected volume of the enzyme solution will have a substantial impact on the reaction and thus the reaction products formed. In the sandwich injection mode, repeatability could be compromised since we inject twice a nanoliter amount of the enzyme solution.

Fig. 2A shows a typical electropherogram after at-inlet reaction of ACE and HHL, introduced by sandwich injection mode; the generated product HA always gave a single peak and no peak splitting was observed.

For six consecutive analyses (sandwich injection, at-inlet reaction), the RSD value was determined to be 4.9% ($n=6$, with a 0 min waiting time, see Section 3.5). The RSD value determined under identical conditions, but with an at-inlet waiting period of 1 min, was determined to be 5.6% ($n=6$). The repeatability of the corrected peak area was acceptable in view of the fact that this parameter is not only affected by the repeatability of the injection of the enzyme solution and of the electrophoretic analysis but also by the enzymatic reaction itself.

Table 2
Within-day repeatability of quantitation of hippuric acid (HA), generated in-capillary

Corrected peak area of HA	RSD, % ($n=6$)	
	0 min wait	1 min wait
Sandwich injection, reaction at capillary inlet (long-end injection)	4.9	5.6
Sandwich injection, reaction at the opposite side (short-end injection)	7.9	7.0

3.4. Reaction at the opposite side (after short-end injection)

In the short-end procedure, enzyme and substrate were injected at the opposite side of the capillary and thus reaction occurred at the part nearest the detector. After a waiting period, the reaction compounds are separated upon application of a voltage (reverse polarity at −6 kV). Here separation is performed in the short distance between the regular capillary outlet and the detector.

As can be seen in Fig. 2B, the electrophoresis run is reduced to a 5 min analysis time. However, the total analysis time, comprising rinse of capillary, injection steps, waiting period and separation, is not reduced accordingly since all the other steps remain unchanged. As shown in Table 2, RSD values are higher compared to the at-inlet reaction. For six consecutive analyses, a RSD value of 7.9% was obtained ($n=6$, with a 0 min waiting time). Unfortunately, we do not have a straightforward explanation for this difference in repeatability. Although this approach was successful, we chose to continue with the at-inlet reaction approach.

3.5. Investigation of waiting period

Following Michaelis–Menten kinetics, we assume that what is being measured is the initial rate v of formation of products: ‘‘when the products have not significantly accumulated and the substrates have not been appreciably depleted’’ [32]. For all enzyme and

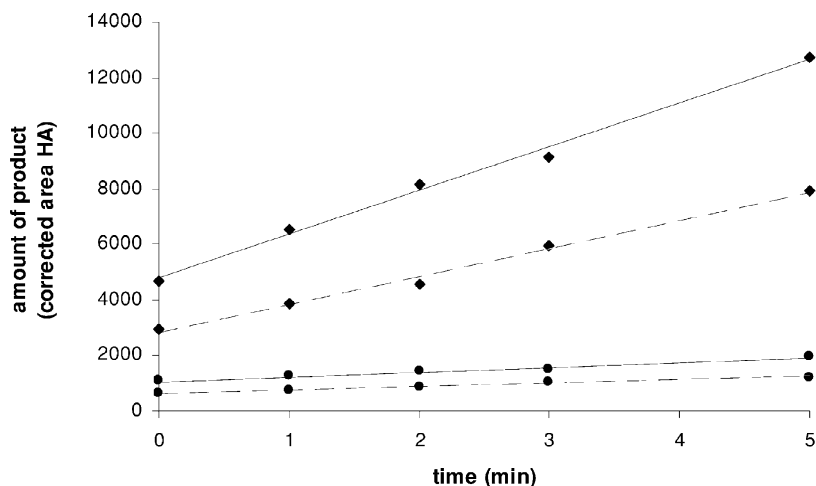


Fig. 3. Reaction velocity (corrected peak area of HA) against in-capillary waiting time, after at-inlet reaction for different combinations of ACE and HHL: ♦___0.42 U/ml ACE, 5.6 m*M* HHL; ♦_ _ _ 0.25 U/ml ACE, 5.6 m*M* HHL; •___0.42 U/ml ACE, 0.14 m*M* HHL; •_ _ _0.25 U/ml ACE, 0.14 m*M* HHL. CE conditions: 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.

substrate concentrations used, the reaction proceeded linearly with time (correlation coefficients at least 0.98). Fig. 3 shows the ACE reaction velocity (corrected peak area of HA) expressed against waiting time, for four different enzyme–substrate combinations.

Although the first point represents a waiting time of 0 min, product formation can already be seen in Fig. 3 at this point. The movements of the P/ACE MDQ robot are slow, the equipment needs about 2–3 min to finish the injection protocol and to bring the vials into run position. Therefore, a waiting time of 0 min means that no additional waiting period was applied. The versatility of the method is illustrated here, since sensitivity can be increased by increasing the waiting period to at least 5 min with in-capillary accumulation of product.

3.6. Michaelis–Menten analysis

For many enzyme-catalyzed reactions, the relation between initial reaction velocity (v) and substrate concentration [S] can be described by the Michaelis–Menten equation [32]:

$$V = \frac{[S]V_{max}}{[S] + K_M} \quad (2)$$

where V_{max} is the maximum reaction velocity and K_M is the Michaelis constant, the substrate concentration at half the maximum velocity. By inversion of this equation, the Lineweaver–Burk plot is obtained, which describes a linear relation between $1/v$ and $1/[S]$.

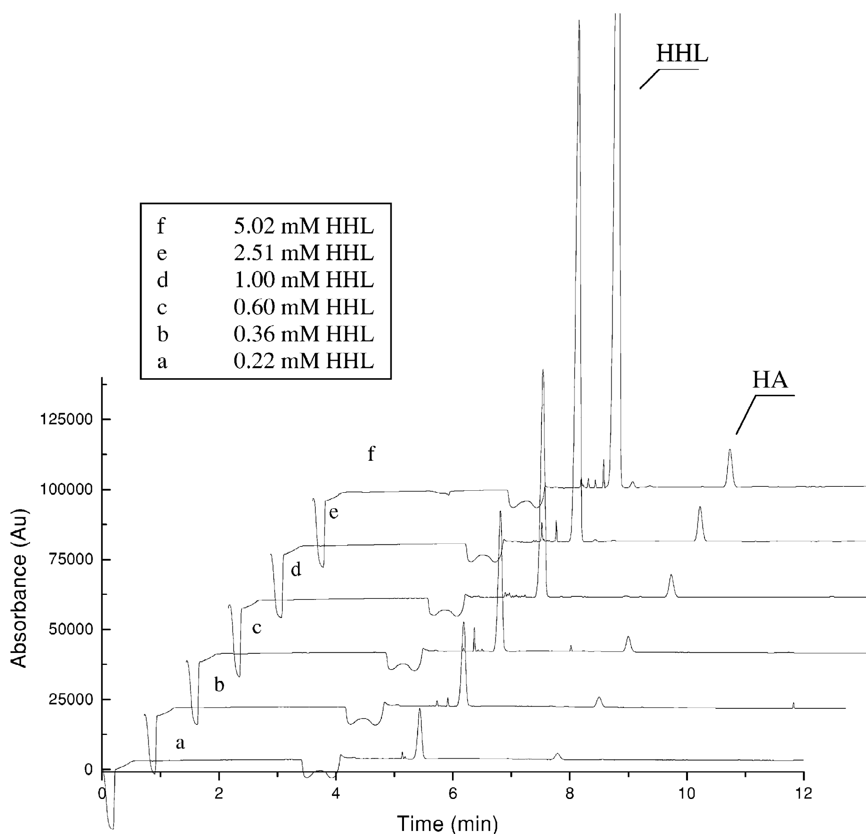


Fig. 4. Overlay of six electropherograms after in-capillary reaction at the capillary inlet between a 0.25 U/ml ACE solution and variable HHL concentration, ranging from 0.22 to 5.02 m*M*. A waiting period of 0 min was applied. CE conditions: run buffer: 150 m*M* HEPES (pH 8.0), applied voltage: 6 kV; current: 76 μA; detection at 230 nm; capillary cartridge temperature: 37 °C.

Six different HHL concentrations, ranging from 0.22 to 5.02 m*M* were used, each concentration analyzed in triplicate. Fig. 4 shows an overlay of six electropherograms, where substrate concentration was varied from 0.22 to 5.02 m*M* for an ACE concentration of 0.25 U/ml.

The Michaelis constant for HHL metabolism was estimated by linear regression from Lineweaver–Burk plots or double reciprocal plots. The K_M values for ACE determined for three different enzyme concentrations (0.42, 0.33 and 0.25 U/ml) were 1.11, 1.08 and 1.29 m*M*, representing an average K_M value of 1.16±0.12 m*M* ($n=3$, RSD 10.2%). The correlation coefficients (r) were 0.9998, 0.9963 and 0.9997, respectively. Fig. 5 shows the Lineweaver–Burk plots for the ACE-catalyzed reactions at the three different ACE concentrations investigated.

In the method employed here, the plugs of enzyme and substrate are introduced on the capillary by consecutive pressure injections and are allowed to stand (and react) during a predetermined waiting period. During this period the plugs will gradually mix, which will cause a dilution of the substrate solution over time. This is a distinctive difference with EMMA-based methods, in which mixing is obtained by electrophoresis without dilution. The fact that no peak splitting was observed for the product peak, could be an indication of complete mixing.

Unfortunately, little is known about the mixing process in such narrow tubes. As a consequence, the mixing process should lower the actual concentrations of the substrate less than nominal concentrations and this could raise the K_M values determined by an unknown factor of the dilution ratio.

Therefore, the Michaelis constant for ACE with HHL as a substrate, cannot be compared readily with K_M values from other studies, since they employed different methods. Furthermore, K_M values vary upon the origin of the ACE enzyme and the exact assay conditions. Reported values range from 0.2 to 2.6 m*M* [7,6,13,29]. Nevertheless, the 1.3 m*M* K_M value for ACE from dogs reported by Meng et al. [13] and the K_M value of 1.1 m*M* for rabbit lung ACE reported by Schnaith et al. [7], are similar to the K_M values reported here.

Future work will be directed towards the investigation of inhibitory compounds in the developed in-capillary system.

3.7. Variation of enzyme concentration

If such a high concentration of substrate is used that only a negligible loss occurs during the assay, a linear relationship of v to the total enzyme concentration can be derived. Five different enzyme concentrations ranging from 0.08 to 0.42 U/ml were

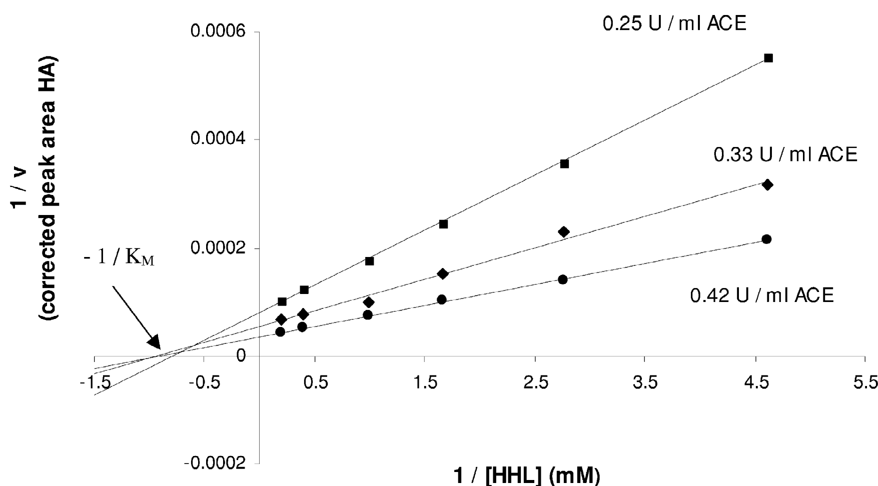


Fig. 5. Lineweaver–Burk plots, for three different ACE concentrations (0.25, 0.33 and 0.42 U/ml); the concentration of HHL varied between 0.22 and 5.02 m*M*. Each concentration was analyzed in triplicate. In-capillary reaction at capillary inlet, waiting period of 0 min. For CE conditions see Fig. 4.

analyzed. The ACE activity of the different enzyme solutions was determined by the corrected peak area of HA, after at-inlet reaction. The substrate concentration was kept constant at 5 m*M* and a waiting period of 1 min was applied. Each solution was analyzed in triplicate and a straight line could be drawn by linear regression analysis. In the regression equation, y is the corrected peak area of the in-capillary generated HA and x represents the enzyme concentration in U/ml ($y = 13707x + 20$; $S_{y,x} = 120$). The correlation coefficient, r, was calculated to be 0.9983. Therefore, the in-capillary reaction proceeded linearly with enzyme concentration.

4. Conclusion

ACE activity was determined by in-line CE after reaction between the enzyme and its tripeptide substrate HHL at the capillary inlet. The corrected peak area of the generated product, HA, was measured spectrophotometrically. Enzyme and substrate were not mixed electrophoretically prior to reaction at the capillary inlet. The whole procedure can also be performed at the opposite side of the capillary, resulting in shorter run times but higher RSD values. By using the at-inlet reaction procedure, a kinetic study of the enzymatic reaction was performed, which yielded a K_M value consistent with previously reported data.

Numerous advantages arise from the described technique such as: (a) minimal reagent use, (b) no need for expensive radioactive materials, and (c) full automation.

The capillary is used as a microvessel and therefore all the different assay steps (incubation, separation, quantitation) are combined in a microscale activity assay. Complete automation reduces the need for human manipulation, lowering the costs and minimizing the risk of cross contamination.

Acknowledgements

S.V.D. is a recipient of a fellowship of the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT).

References

[1] J.S. White, D.C. White (Eds.), Source Book of Enzymes, CRC Press, New York, 1997, p. 516.
[2] B. Bénéteau-Burnat, B. Baudin, Crit. Rev. Clin. Lab. Sci. 28 (1991) 337.
[3] J. Lieberman, T.H. Rea, Ann. Intern. Med. 87 (1977) 422.
[4] D.W. Cushman, M.A. Ondetti, Biochem. Pharmacol. 29 (1980) 1871.
[5] P. Bünning, J. Card. Pharmacol. 10 (1987) S31.
[6] D.W. Cushman, H.S. Cheung, Biochem. Pharmacol. 20 (1971) 1637.
[7] E. Schnaith, R. Beyrau, B. Bückner, R.M. Klein, W. Rick, Clin. Chim. Acta 227 (1994) 145.
[8] J.W. Ryan, A. Chung, C. Ammons, M.L. Carlton, Biochem. J. 167 (1977) 501.
[9] K.W. Ryder, H. Thompson, D. Smith, M. Sample, R.B. Sample, T.O. Oei, Clin. Biochem. 17 (1984) 302.
[10] J. Friedland, E. Silverstein, Am. J. Clin. Pathol. 66 (1976) 416.
[11] S.G. Chiknas, Clin. Chem. 25 (1979) 1259.
[12] H.M. Neels, S.L. Scharpé, M.E. van Sande, R.M. Verkerk, K.J. Van Acker, Clin. Chem. 28 (1982) 1352.
[13] Q.C. Meng, E. Balcells, L. Dell'italia, J. Durand, S. Oparil, Biochem. Pharmacol. 50 (1995) 1445.
[14] Z.K. Shihabi, J. Chromatogr. A 853 (1999) 185.
[15] R.-Z. Zang, X.-H. Xu, T.-B. Chen, L. Li, P.-F. Rao, Anal. Biochem. 280 (2000) 286.
[16] J. Bao, F.E. Regnier, J. Chromatogr. 608 (1992) 217.
[17] K.J. Miller, I.K. Leesong, J.M. Bao, F.E. Regnier, F.E. Lytle, Anal. Chem. 65 (1993) 3267.
[18] D. Wu, F.E. Regnier, M.C. Linhares, J. Chromatogr. B 657 (1994) 357.
[19] J.J. Bao, J.M. Fujima, N.D. Danielson, J. Chromatogr. B 699 (1997) 481.
[20] T. Watanabe, A. Yamamoto, S. Nagai, S. Terabe, Electrophoresis 19 (1998) 2331.
[21] J. Saevels, A. Van Schepdael, J. Hoogmartens, Electrophoresis 17 (1996) 1222.
[22] S. Van Dyck, A. Van Schepdael, J. Hoogmartens, Electrophoresis 22 (2001) 1436.
[23] S. Van Dyck, A. Van Schepdael, J. Hoogmartens, Electrophoresis 23 (2002) 2854.
[24] J. Saevels, K. Van den Steen, A. Van Schepdael, J. Hoogmartens, J. Chromatogr. A 745 (1996) 293.
[25] A.R. Whisnant, S.E. Johnston, S.D. Gilman, Electrophoresis 21 (2000) 1341.
[26] A. Taga, S. Honda, J. Chromatogr. A 742 (1996) 243.
[27] R. Kuhn, S. Hoffstetter-Kuhn, Capillary Electrophoresis: Principles and Practice, Springer, Berlin, 1993, p. 43, p. 232.
[28] B.-W. Chang, R.L. Chen, I.-J. Huang, H.-C. Chang, Anal. Biochem. 291 (2001) 84.
[29] J.R. Schullek, I.B. Wilson, Peptides 10 (1989) 431.
[30] Z.K. Shihabi, J. Chromatogr. A 902 (2000) 107.
[31] S. Van Dyck, A. Van Schepdael, J. Hoogmartens, Electrophoresis 23 (2002) 1341.
[32] A. Fersht, in: Structure and Mechanism in Protein Science, W.H. Freeman, New York, 1999, p. 104.