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SENSITIVE METHOD FOR QUANTITATION OF ANGIOTENSIN-CONVERTING ENZYME (ACE) ACTIVITY IN TISSUE

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Abstract—A novel sensitive and specific method for the measurement of tissue angiotensin-converting enzyme (ACE) activity utilizing HPLC is described. ACE activity was determined in detergent-extracted canine hearts utilizing the synthetic ACE-specific substrate hippuryl histidyl leucine (HHL), both in the presence and the absence of the site-specific inhibitor captopril. Tissue ACE activity was quantitated from the moles of hippuric acid (HA) formed, in time-fixed assays, utilizing HPLC separation of HA from HHL and UV-spectrophotometry for quantitation of HA as in the standard Cushman and Cheung assay (Cushman DW and Cheung HS, *Biochem Pharmacol* **20**: 1637–1648, 1971). Separation of HA from HHL was performed by reverse phase HPLC on a phenyl silica gel column with an eluent consisting of 20% acetonitrile in 0.1 M aqueous ammonium phosphate buffer, pH 6.8. After the standard liquid/liquid extraction procedure with ethyl acetate, HPLC analysis revealed the presence of unreacted substrate, HHL, in amounts comparable to the product of interest, HA, in the final assay; moreover, the amount of HA formed did not fall completely to zero in the presence of captopril. Regional studies of canine cardiac ACE activity utilizing the HPLC-based assay and the standard assay method showed a significantly higher ACE activity in the right ventricle compared with the left ventricle (2.37 ± 0.7 vs 1.24 ± 0.18 mU/g, $P < 0.05$ [$N = 6$], respectively) in the HPLC-based assay, but no difference in right and left ventricular ACE activities by the standard assay (0.25 ± 0.08 vs 0.31 ± 0.09 mU/g [$N = 6$], respectively). Kinetic studies utilizing the HPLC-based assay coupled with the use of captopril showed K_m (1.34 ± 0.08 mM) and V_{max} ($36.8 \pm 11.5 \times 10^{-10}$ M/min) values in agreement with those in the literature. Our results demonstrate that the application of HPLC to the standard Cushman and Cheung assay improves the sensitivity and specificity of the standard assay and enables the use of much smaller amounts (~4 vs ~400 mg for the Cushman and Cheung assay) of tissue for ACE activity assay.

Key words: angiotensin-converting enzyme; high performance liquid chromatography; angiotensin I; angiotensin II; renin-angiotensin system

ACE§ is a dipeptidyl carboxypeptidase (EC 3.4.15.1) whose physiologic action is the conversion of AI to AII, and as such it plays an important role in the regulation of blood pressure and fluid balance. ACE has been localized to endothelial cells throughout the body and epithelial cells in gut and kidney. It exists both as a membrane-bound enzyme and in a freely soluble form in plasma. The importance of the membrane-bound form is underscored by the recently described local RAS in various organs, including heart, blood vessels and kidney [1]. In the heart and vascular smooth muscle, the local RAS is believed to play an important role in hypertrophy and remodeling. Accordingly, accurate and reliable methods for measurement of tissue (i.e. membrane bound) ACE activity are important.

Several methods for the measurement of ACE activity have been described, with that of Cushman and Cheung [2] being most commonly utilized. This method uses the ACE-specific substrate HHL coupled with spectrophotometric detection of the product HA. Other methods for quantitating ACE activity utilize bioassay [3], radioisotopic [4, 5], fluorometric [6] and HPLC procedures [7, 8] for measurement of the product. These assays have low sensitivity and/or reproducibility due to incomplete inhibition of the enzyme and/or the presence of interfering substances, resulting in high blank values. In addition, most methods for ACE activity measure the soluble form of the enzyme. Membrane-bound ACE activity in tissues has been quantitated utilizing trypsin pretreatment to remove the active enzyme from the cell membranes. Trypsin extraction removes the membrane binding sequences from ACE and thus produces subtle alterations in the size and charge of the molecule [9]. Whether these effects alter the catalytic activity of the enzyme is uncertain. Standard detergent extraction procedures may result in incomplete extraction of ACE from membranes. In addition, they may cause high blank values in the spectrophotometric ACE assay because of difficulty in removing the detergent, which interferes with the spectrophotometric quantitation of HA.

Accordingly, we have developed a sensitive method for the accurate and reproducible quantitation of membrane-bound ACE activity in tissue, in which ACE is extracted with detergent and the reaction product is isolated from the reaction mixture by reverse phase HPLC,

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§ Abbreviations: ACE, angiotensin-converting enzyme; HHL, hippuryl histidyl leucine; HA, hippuric acid; AI, angiotensin I; AII, angiotensin II; RAS, renin-angiotensin system; and CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-I-propanesulfonate.

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thus eliminating interference from the detergent and reaction byproducts. This method is a modification of the Cushman and Cheung procedure, which utilizes the artificial substrate HHL and quantitates the product HA by UV detection at 228 nm. The active site-specific ACE inhibitor captopril is used to inhibit the enzyme in blank samples and increase the specificity of the assay.

MATERIALS AND METHODS

Animals

Healthy mongrel dogs of both sexes weighing 18–25 kg (N = 6) underwent induction of a deep surgical plane of anesthesia with isoflurane inhalation anesthesia, and then were subjected to thoracotomy. The heart was arrested with a lethal dose of KCl and removed from the chest, rapidly cooled in ice-cold phosphate buffer, and placed on a stainless steel tray on ice. The heart and lung were then dissected, and tissue (right and left atria, right and left ventricles [apex, mid, base], and septum and both lungs en bloc) was flash frozen in liquid N_2 and stored at −80°. Dissection of heart was performed carefully excluding endo- and epicardium from tissue samples. All procedures followed in these experiments are in accordance with institutional guidelines and were approved by the University of Alabama at Birmingham Animal Use Review Committee.

ACE activity assay—With HPLC separation

Tissues were homogenized in 0.02 M potassium phosphate buffer (pH 8.3), centrifuged at 40,000 *g* for 20 min at 4°, and washed three times with the same buffer. The final tissue pellet was resuspended to a concentration of 0.2 g/mL in 0.1 M phosphate buffer; from this 50 µL of homogenized tissue was removed for assay. Tissue extracts (50 µL) were incubated with 500 µL of a reaction mixture containing 0.3 M NaCl, 0.01% Triton X-100, 10^{-4} M $ZnCl_2$ and 5 mM HHL as substrate in 0.1 M phosphate buffer, pH 8.3, at 37° for 30 min. A second 50-µL tissue aliquot was preincubated with 0.1 mM captopril for 30 min at room temperature prior to the addition of HHL as a tissue blank in order to define specific ACE activity. The enzymatic reactions were terminated by addition of 500 µL of 1 N HCl. The HA formed by the action of ACE on HHL (500 µL) was extracted from the acidified solution into 1.5 mL of ethyl acetate by vortex mixing for 15 sec. After brief centrifugation, a 1-mL aliquot of each ethyl acetate layer was transferred to a clean tube and dried by heating at 120° for 30 min. The dried HA samples were redissolved in 0.5 mL of the HPLC mobile phase, which contained 15% acetonitrile (CH_3CN) in 0.1 M ammonium phosphate buffer (v/v), pH 6.8, and applied to a reverse phase Alltima 5 µm-phenyl HPLC column (Alltech Associates, Deerfield, IL).

HPLC separation of HHL and HA

The HPLC system consisted of two model 501 pumps, a Type U6K injection valve equipped with a 1-mL sample loop, a µBondapak phenyl pre-column, an Alltima phenyl analytical column (250 mm × 4.6 mm i.d., 5 µm particle size), a model 404 tunable UV detector (all from Waters Associates, Milford, MA) set at 228 nm, and a one-channel recorder (Kipp and Zonen, BD 40, Alltech Associates). The HPLC separation of HHL and HA was carried out at 35° at a flow rate of 1.0 mL/min. The protein content of the samples was determined according to Lowry *et al.* [10]. Values for protein content of samples were tightly correlated with wet weights of the tissues (10 µg protein/mg wet weight of heart tissue). Therefore, all data were expressed as units of HA formed per gram tissue wet weight (1 U = 1 µmol HA formed/min at 37°). The intraassay and interassay coefficients of variation of a standard sample of dog lung were 3.5% (N = 6) and 4.6% (N = 6), respectively.

ACE activity assay—Without HPLC separation

Heart tissue (left and right ventricle) was homogenized in 0.02 M potassium phosphate buffer (pH 8.3), centrifuged at 40,000 *g* for 20 min at 4° and washed three times with the same buffer. The final pellet, or membrane fraction, was retained and dissolved in 5 mM CHAPS overnight at 4°. This extraction procedure has been shown to be as efficient as Triton X-100 extraction for tissue ACE because a prolonged period is allowed for CHAPS to extract the enzyme from the membrane [9]. Note that the period of incubation with Triton X-100 in our assay is only 30 min, thus expediting the assay procedure. Thereafter, tissue aliquots were incubated with a reaction mixture containing 0.3 M NaCl, 10^{-4} M ZnCl and 5 mM HHL as substrate in 0.1 M phosphate buffer, pH 8.3, at 37° for 30 min. A second reaction mixture was preincubated with 0.1 mM captopril for 30 min at room temperature prior to the addition of HHL as a tissue blank in order to define specific ACE activity. The enzymatic reactions were terminated by addition of 500 µL of 1 N HCl. From this mixture 500 mL was extracted with 1.5 mL of ethyl acetate. The HA formed by the action of ACE on HHL (500 µL) was extracted from the acidified solution into 1.5 mL of ethyl acetate by vortex mixing for 15 sec. After brief centrifugation, a 1-mL aliquot of each ethyl acetate layer was transferred to a clean tube and dried by heating at 120° for 30 min. The dried HA samples were redissolved in distilled H_2O and read in a Shimadzu UV recording spectrophotometer at 228 nm. Data were calculated using the method of Cushman and Cheung [2].

Recovery experiment

Recovery of HA from the ethyl acetate liquid extraction procedure was determined from known molar quantities of standard HA solutions subjected to the same conditions as the tissue assay. Briefly, a 5-mM solution of HA was prepared in 0.01% Triton X-100, 0.02 M phosphate buffer (pH 8.3); 500 µL was transferred into a dry test tube and combined with 500 µL of 1 N HCl. The mixture was agitated on a vortex-mixer for 30 sec and centrifuged at 3000 *g* for 20 min; then 500 µL of the solution was transferred to a tube containing 1.5 mL of ethyl acetate and vortexed. After centrifugation, 1 mL of the upper organic layer was transferred to a tube and dried by heating at 120° for 30 min. The extracted samples were injected into the HPLC under the conditions described above, and recovery of HA was calculated from the ratio of measured HA to the amount injected.

Recovery of HA from detergent-extracted samples was 97% with the HPLC separation/detection method compared with 91% reported in the literature for the standard procedure of Cushman and Cheung [2].

Kinetic studies

Dog lung tissue was used for kinetic studies of tissue ACE activity utilizing the HPLC assay protocol. Tissue

was prepared and ACE activity determined as described above utilizing the ACE-specific substrate HHL and the active site-directed ACE inhibitor captopril. K_m and V_{max} were determined according to the method of Eisenthal and Cornish-Bowden [11]. Briefly, initial velocities (V_o) at various initial substrate concentrations (S_o) were determined from the initial slopes of their corresponding time-dependent studies. Direct linear plots of V_o against S_o were then generated, defining a family of lines. K_m and V_{max} were determined from median values between the points of intersection (of these lines) projecting onto the abscissa and ordinate, respectively.

Data analysis

Peaks for HA and HHL were identified by comparison with the retention times of standard compounds. Standard solutions of HA (Sigma Chemical Co., St. Louis, MO) were prepared daily. A solution of 10 μg/mL HA was prepared in the HPLC mobile phase and diluted serially to provide calibration standards.

Statistics

Values are expressed as means ± SEM. Student's unpaired *t*-test was used for comparisons between groups. Differences were considered to be statistically significant at the $P < 0.05$ level.

RESULTS

Under the isocratic conditions described above, a clear separation of HA from HHL was achieved for standard compounds (Fig. 1, left panel). Approximately 10 ng of HA and of HHL were applied to the column. A representative chromatogram obtained in an assay of ACE activity in a normal dog left ventricle is shown in the middle panel of Fig. 1. Note that the HA peak is easily quantifiable and that a large HHL peak is present. The retention times of these peaks were identical with those of the standard compounds. In addition, spiking samples with standard HA (0.1 ng) resulted in increased amplitude of the HA peak, confirming the identity of the compound. The identity of the small peaks is unknown. The

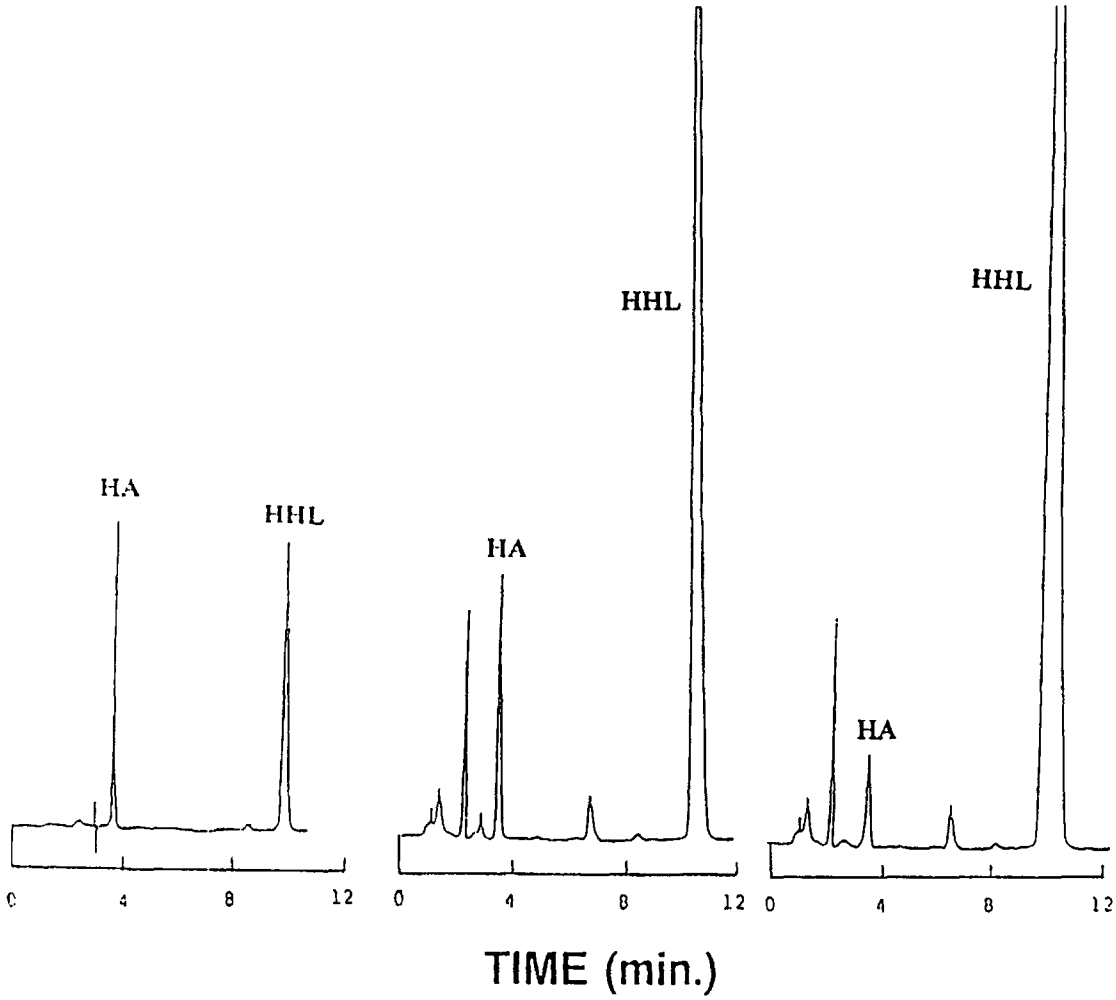

Fig. 1. HPLC chromatograms. Left panel: Chromatogram for 0.01 μg hippuric acid (HA) and hippuryl histidyl leucine (HHL) standards. Middle panel: Chromatogram from dog left ventricle assay (1 mg of midwall) showing HA peak and persistent substrate (HHL) peak following a 40-μL HPLC injection from the final reaction product after extraction. Right panel: Chromatogram from dog left ventricle assay in the presence of 0.1 mM captopril following a 90-μL HPLC injection from the final reaction product after extraction.

right panel of Fig. 1 depicts the results of adding captopril (10^{-4} M) to the reaction mixture: generation of HA was clearly reduced, but not to zero, with a corresponding increase in the substrate (HHL) peak. Indeed, 37 ± 3.4% of HA formation was not inhibited by captopril. Standard curves were used to quantitate the amount of HA formed and were linear over the concentration range studied (Fig. 2).

ACE activity assay was performed by the standard Cushman and Cheung method and our HPLC-based method on aliquots of the left ventricle of the same dog. In the standard methodology, CHAPS was used to extract membranes because non-dialyzable Triton X-100 interferes with the spectrophotometric assay. Figure 3 shows the comparison between the Cushman and Cheung (standard) and HPLC-based (new) assays in dog left ventricle (midwall). ACE activity measured with the HPLC-based assay was 1.24 ± 0.18 mU/g (N = 6), 4-fold the values obtained with the standard Cushman and Cheung assay (0.31 ± 0.09 mU/g, N = 6). ACE activity measurements in right ventricle and midwall of left ventricle of six normal dogs by both HPLC and standard Cushman and Cheung methods are summarized in Fig. 4. The HPLC-based analysis revealed a significant difference (right > left) in ACE activity between the ventricles; this regional difference in ACE expression was not evident when ACE activity was measured by the unmodified Cushman and Cheung technique. Our observation of a 2-fold increase in ACE activity in the right ventricle compard with the left confirms the previous report of Urata [12] in normal and failing human hearts. Further, ACE activity when measured by the HPLC assay was greater (4- and 10-fold, respectively) in both left and right ventricles than when measured by the Cushman and Cheung assay.

Values for K_m (1.34 ± 0.08 mM) and V_{max} (36.8 ± 11.5 × 10^{-10} M/min) showed that the assay characteristics are in agreement with published results obtained using purified preparations of ACE (Fig. 5) [9]. Utilizing our HPLC method, the time dependence of tissue ACE activity was linear out to 30 min (Fig. 6).

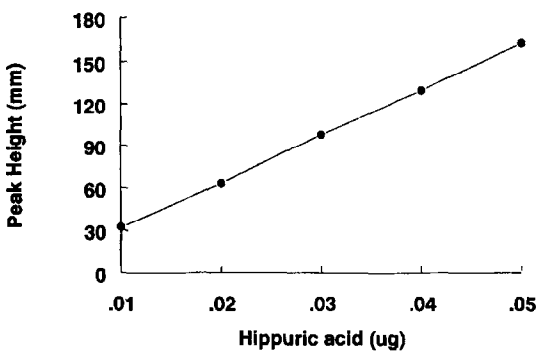

Fig. 2. Standard curve for hippuric acid from HPLC chromatograms.

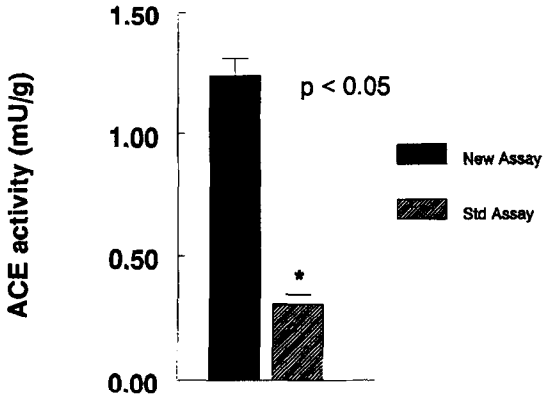

Fig. 3. Comparison of HPLC-based assay (new) with standard Cushman and Cheung assay on tissue from dog left ventricle (midwall). Results are means ± SEM, N = 6 for each group. The asterisk (*) indicates a significant difference between the new and the standard assays ($P < 0.05$).

DISCUSSION

The accurate measurement of both membrane bound and soluble ACE activity is important, as tissue ACE activity is involved in a number of pathophysiologic states, including hypertension and myocardial hypertrophy, and ACE inhibitors are therapeutically useful in these disease states. Several methods exist for the determination of ACE activity, with most relying on synthetic substrates in order to define ACE activity without confounding by the concomitant activity of angiotensinases. ACE activity assays based on the standard Cushman and Cheung method have limited sensitivity because of the presence of interfering substances that persist after the extraction procedure. We have utilized HPLC coupled with the ACE-specific substrate HHL and the ACE-specific inhibitor captopril in order to determine tissue ACE activity in the normal canine heart. Our results demonstrate that the persistence of unreacted HHL substrate can greatly limit both the sensitivity and specificity of the standard Cushman and Cheung procedure. The finding of a very large HHL peak on HPLC in the assay of a representative sample of dog left ventricle indicates incomplete removal of the HHL substrate during the standard liquid/liquid extraction procedure. In the ab-

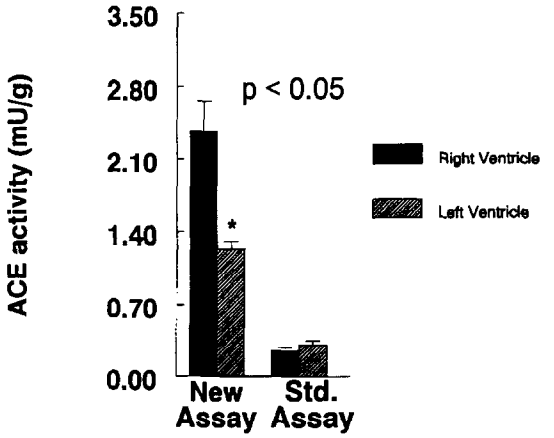

Fig. 4. Tissue ACE activity heterogeneity in normal canine hearts (left ventricle and right ventricle); comparison between HPLC-based assay (left bars) with standard Cushman and Cheung assay (right bars). Results are means ± SEM, N = 6 for each group. The asterisk (*) indicates a significant difference between values for right and left ventricle by the HPLC assay ($P < 0.05$).

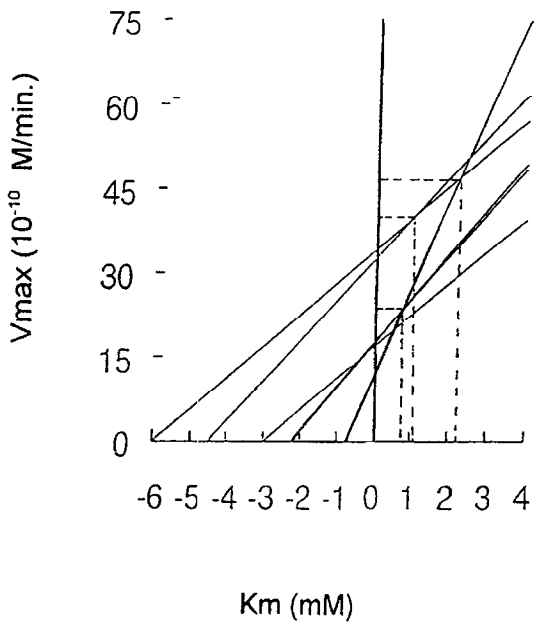

Fig. 5. Direct linear plot for canine lung ACE activity as determined by the HPLC assay. K_m and V_{max} were determined by averaging the corresponding values from the points of intersection between the dashed lines and the abscissa and ordinate, respectively.

sence of HPLC separation, as in the standard Cushman and Cheung assay, this can create interference of greater magnitude than the value for the HA generated in the reaction, yielding negative values for ACE activity. HPLC separation of HA from HHL eliminates this interference and increases the sensitivity of the assay. In addition, HPLC permits the use of stronger detergents for the extraction of ACE from membranes, improving the efficiency of removal of the enzyme from tissue, minimizing incubation times, and increasing the sensitivity of the assay. Our comparison between the Cushman and Cheung procedure and our HPLC-based assay

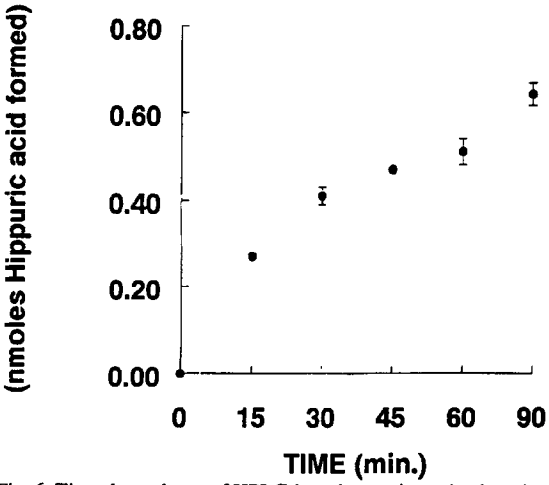

Fig. 6. Time dependence of HPLC-based assay in canine lung in the presence of 1.5 mM HHL. Each point is the mean ± SEM of three determinations.

demonstrated that improved sensitivity results from efficient removal of the interfering substrate HHL and the detergent Triton X-100. A doubling in sensitivity was observed by our method from protein content determinations. Only 40 μg of protein, extracted from ~4 mg of heart tissue, was required for our HPLC-based assay, as opposed to 4 mg of protein, extracted from 400 mg of heart tissue, for the standard Cushman and Cheung assay.

To improve the specificity of our method, we utilized the site-specific ACE inhibitor captopril to inhibit the enzyme in blank samples. As is evident in Fig. 1, the generation of HA was not inhibited completely by captopril, indicating the possibility of alternative HA-generating pathways, such as heart chymase-like activity, as demonstrated in the human heart [12]. Thus, generation of HA in the standard Cushman and Cheung procedure is not entirely specific for ACE. Our demonstration of K_m and V_{max} values in the HPLC assay identical to those obtained using pure ACE [9] indicates that our assay is highly specific for ACE.

There is a large body of clinical data demonstrating the beneficial effects of ACE inhibitors in reducing mortality, rate of recurrent myocardial infarction, and subsequent development of heart failure in patients after myocardial infarction [13, 14] and in patients with chronic congestive heart failure [15–17]. These effects cannot be explained by blood pressure reduction alone [18]. This clinical information, coupled with the evidence for the existence of a functional intracardiac renin-angiotensin system in the rat and of ACE gene polymorphisms that are associated with increased risk of myocardial infarction and cardiomyopathy in humans, has led to the hypothesis that the intracardiac renin-angiotensin system is activated locally, while the circulating renin-angiotensin system remains unaltered in patients with asymptomatic left ventricular dysfunction [19, 20]. Thus, an ACE activity assay that combines high specificity with high sensitivity and therefore can be performed on a very small amount of tissue will be very useful in investigating the pathophysiologic role of the cardiac renin-angiotensin system and the mechanism of action of ACE inhibitors at the tissue level.

We have shown that by applying HPLC separation to the standard Cushman and Cheung assay for ACE activity, we were able to increase the sensitivity of the assay, permitting accurate quantitation of ACE activity in 4 to 5-mg samples of normal dog heart. Moreover, by utilizing the ACE-specific inhibitor, captopril, specificity was improved without interfering with the HPLC detection of HA and HHL. This method will be useful in studies of the role of the tissue renin-angiotensin system in the pathogenesis of cardiovascular disease in animal models and humans, as well as in documenting the effects of ACE inhibitor and angiotensin II antagonist therapy on these processes.

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