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Simultaneous separation of angiotensin and endothelin peptide families by high-performance liquid chromatography: application to the specific radioimmunoassay measurement of angiotensin II or endothelin-1 from tissue

George O.A. Naik*, Gordon W. Moe

St. Michael's Hospital and the Department of Medicine, University of Toronto, Toronto, ON, Canada

Abstract

Currently available measurements of endogenous angiotensin II (ANG II) and endothelin-1 (ET-1) concentrations by radioimmunoassay (RIA) lack specificity to ANG II or ET-1. ANG II and ET-1 antibodies cross-react with immuno-reactive angiotensin and endothelin family members, respectively. We have therefore developed an ion-pair reversed-phase high-performance liquid chromatography (HPLC) for simultaneously separating angiotensin and endothelin peptides and enhancing RIA specificity in the measurement of ANG II and ET-1. The developed HPLC separation was applied to canine myocardium extracts; ANG II or ET-1 fractions were collected and quantified by RIA. Elution times for both peptide families, ANG I, ANG II, ANG III, ANG IV, ANG II(4–8), bET-1, ET-1, ET-2 and ET-3 were within 25 min. In normal canine myocardium from the right atrium, right ventricle, left atrium and left ventricle, ANG II concentrations were 39±11, 28±21, 31±11 and 21±8 fmol/g and ET-1 concentrations were 43±16, 42±19, 55±21 and 57±34 fmol/g (mean±SD, $N=7$), respectively. The combination of HPLC with RIA renders the measurement of ANG II or ET-1 specific and convenient, and saves time. This HPLC separation may be applied to the specific measurement of other immuno-reactive angiotensin and endothelin peptides.

Keywords: Angiotensins; Endothelins; Peptides

1. Introduction

Angiotensin II (ANG II) and endothelin-1 (ET-1) [1] are potent vasoconstrictor peptides implicated in normal physiology and pathophysiology processes [2,3]. Angiotensinogen, encoded by a single gene, is sequentially cleaved to produce the angiotensin family of linear peptides having ten or fewer amino acid residues. Angiotensinogen is cleaved by renin to angiotensin I (ANG I), which in turn is cleaved by angiotensin converting enzyme to angiotensin II (ANG II). Aspartic acid from the N-terminus of ANG II is cleaved by aminopeptidaseA to angiotensin III (ANG III), which in turn is cleaved by aminopeptidaseB to an arginine residue and angiotensin IV (ANG IV). ANG IV is further transformed to angiotensin II(4–8) (ANG II(4–8)) [4]. Circulating ANG II is an integral participant of blood pressure control and is elevated in hypertension and congestive heart failure (CHF) [5]. Localized tissue production of ANG II is observed in various organs, including the heart. ANG II has vasoconstrictive and

*Corresponding author. Tel.: +1-416-864-5723; fax: +1-416-864-5723.

E-mail address: george.naik@utoronto.ca (G.O.A. Naik)

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trophic effects [5,6], and it stimulates extracellular collagen synthesis [7]. Angiotensin biological effects are mediated by cell surface receptors; ANG II effects are mediated by AT_1 and AT_2 receptors. ANG III is a less potent vasoconstrictor than ANG II. ANG IV, derived from ANG II, has inhibitory trophic and vasodilatory effects, opposite to ANG II [4,8,9], and its action is mediated by a putative receptor, AT_4, which is different from AT_1 and AT_2 receptors.

Endothelin isoforms, endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3), each have 21 amino acid residues with two disulphide bonds to form a noose. They are encoded by different genes. Preproendothelin-1, the translated product of the ET-1 gene, is cleaved by endopeptidases to form big endothelin-1 (bET-1) [10]. Endothelin converting enzyme cleaves bET-1 to form ET-1, similar pathways may be responsible for the production of ET-2 and ET-3. ET-1 is a more potent vasoconstrictor than ANG II. ET-1 is present in the systemic circulation and is elevated in CHF and after myocardial infarction, but not in hypertension [11]. Endothelins, as well as angiotensins, have been observed in different tissues, including the myocardium. Two cell surface receptors, ET_A and ET_B, have been identified through which endothelins effect their biological actions [12]. ET-1 and ET-3 stimulation of ET_B receptor on endothelial cells elicits vasodilatation via the production of nitric oxide and prostacyclin, which in turn relaxes adjacent smooth muscle. However, ET-1 stimulation of ET_A receptor on smooth muscle cells produces vasoconstriction [12].

The angiotensins and endothelin isoforms are genetically regulated and have various biological effects through their respective receptors. It is therefore desirable to have a method that is specific for the measurement of individual peptides, such as ANG II and ET-1, as provided by high-performance liquid chromatography (HPLC) combined with radioimmunoassay (RIA). RIA, which is based on the binding of antigen to antibodies, is widely used in the measurement of endogenous substances. RIA can detect low ANG II or ET-1 concentrations; however, it is not specific for the measurement of either ANG II or ET-1 alone. The ANG II antibody cross-reacts with other members of the angiotensin family, such as, ANG III, ANG IV and ANG II (4–8) [13,14]. Similarly, the ET-1 antibody cross-reacts with other endothelin isoforms, such as, ET-2 and ET-3, and bET-1 [15–17]. Consequently, RIA measurement alone of ANG II or ET-1 not only detects ANG II or ET-1, but additional immunoreactive members within each peptide family.

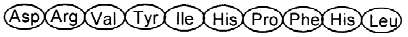
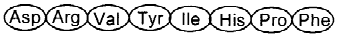
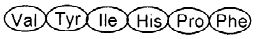
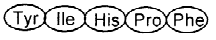
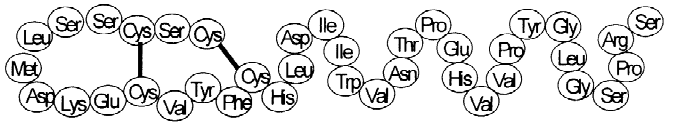
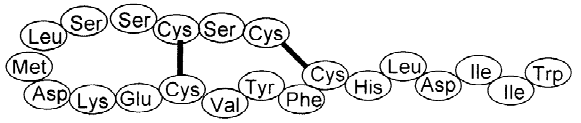
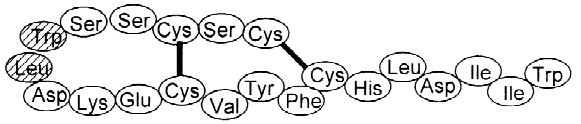
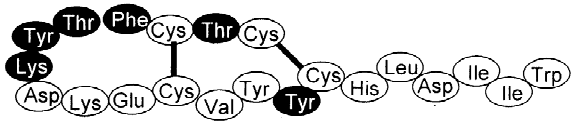
In order to enhance the specificity of ANG II or ET-1 RIA measurement, HPLC has been used to separate immuno-reactive peptides from ANG II or ET-1, respectively [17,18]. HPLC with a reverse phase column rapidly and efficiently separates peptides [19]. Various investigators have combined HPLC with RIA in the measurement of ANG II [17,20–25] or ET-1 [26,27]. To our knowledge, a combined separation, however, of both angiotensins and endothelins in a single HPLC run has not been published; such a combined separation would save time and cost. We initiated efforts to simultaneously separate the angiotensin and endothelin peptide families by ion-pair reverse phase HPLC with triethylammonium formate (TEAF) as an ion-pairing reagent. The angiotensins having a lower molecular weight range of 774–1296 g/mol are expected to elute first, followed by the endothelins with molecular weight range of 2490–4280 g/mol, as shown in Table 1. In order to be time efficient we plan to utilize our HPLC system gradient delay, in which separation is essentially isocratic. By design, we want early eluting peaks to fall within the isocratic region of the chromatogram and late eluting peaks to be under gradient control. Application of the developed HPLC separation to tissue extracts, collection of ANG II or ET-1 fraction, followed by RIA quantification. We also employed a validated two-injector system to minimize HPLC-derived shadowing, as previously reported [28].

2. Experimental

2.1. Peptides and chemicals

ANG I, ANG II, ANG III, (Bachem, Philadelphia, PA, USA); ANG IV (ANG II(3–8)), ANG II(4–8) (Bachem California, Torrance, CA, USA); formic acid (HCOOH) (Sigma, St Louis, MO, USA). HPLC grade solvents: methanol (CH_3OH) and acetonitrile (CH_3CN) (EM Science, Toronto, ON, Canada); triethylammonium formate ($(C_2H_5)_3NHCOOH$,

Table 1
Molecular properties of angiotensin and endothelin peptide families

Peptide name	Amino acid sequence	Mol. wt.[a]	No. a.a[b]
ANG I		1296	10
ANG II		1046	8
ANG III		931	7
ANG IV		774	6
ANG II(4-8)		512	5
bET-1		4280	38
ET-1		2490	21
ET-2		2545	21
ET-3		2641	21

[a] Mol. wt.=Molecular weight in g/mol.
[b] No. a.a=Number of amino acid residues.

TEAF) (Fluka, Ronkonkoma, NY, USA). Polystyrene tubes (Simport Plastics, Beloeil, PQ, Canada) were used.

2.2. Tissue sampling

Normal adult male mongrel dogs were anesthetized with intravenous sodium thiopental 25 mg/kg and ventilated with room air using a ventilator (CCV-2 Ohio Critical Care). A left thoracotomy was performed and cardiac tissue samples were obtained. Excised tissue was quickly frozen in liquid nitrogen and then stored at −80°C until time of assay. Approval was obtained from the Animal Care and Use Committee of St. Michael's Hospital Health Sciences Research Centre.

2.3. Tissue extraction and solid-phase extraction

2.3.1. Angiotensins

Angiotensin extraction from tissue has been previously described [29]. Tissue samples were thawed at 4°C, cleaned, and homogenized with a Polytron (Brinkmann Instruments, Streetsville, ON, Canada)

in 20 volumes of ice-cooled 0.18 *M* HCl–ethanol (1:3 v/v). The homogenate was centrifuged at 32 500 *g* (Model L8-80, Beckman, Mississauga, ON, Canada) with a 50.3 Ti rotor (Beckman, Mississauga, ON, Canada) for 20 min at 4°C. The supernatant was separated from the pellet and its pH was adjusted to between 5.5 and 6.0 with 1 *M* NaOH. The supernatant was kept on ice for 1 h and spun at 2200 *g* (OmnifugeRT, Baxter, Mississauga, ON, Canada) for 10 min at 4°C. The pH was measured again and adjustments were made if necessary with 1 *M* NaOH and/or 0.18 *M* HCl and was spun again at 2200 *g* for 20 min at 4°C. The decanted supernatant was evaporated in a vacuum concentrator (Savant, Farmingdale, NY, USA) and was stored at −20°C.

A solid-phase SepPak C_{18} column (Waters, Mississauga, ON, Canada) was conditioned with 6 ml each of methanol and then with deionized water. The dried supernatant was reconstituted with 2×500 μl of 1 *M* formic acid and was applied to a preconditioned SepPak. The loaded SepPak column was washed with 6 ml of 10% methanol in 1 *M* formic acid. Angiotensins were eluted with 8 ml of 80% methanol in 1 *M* formic acid, and the eluent dried in a vacuum concentrator.

2.3.2. Endothelins

Normal canine myocardium tissue was homogenized in 1 *M* acetic acid (1:10) using a Polytron. Homogenate was centrifuged at 31 000 *g* for 30 min at 4°C. The supernatant was solid-phase extracted with SepPak C_{18} columns. Loaded columns were washed with 3 ml of 10% methanol in 1.0 *M* formic acid. Endothelins were eluted with 80% methanol in 1.0 *M* formic acid. The eluate was dried under vacuum using a Speed-Vac and was stored at −20°C until HPLC separation. Before injection onto the HPLC column, the dried eluate was reconstituted with mobile phase and filtered with Ultrafree-MC filters (Millipore, Bedford, MA, USA).

2.4. High-performance liquid chromatography

2.4.1. Method development rationale

Linear gradient delay of our HPLC system at a flow-rate of 1.2 ml/min was about 9 min, i.e., the time it takes buffer A and B to mix and to be detected. We wanted early eluting peaks to fall within the isocratic region (during the gradient delay) of the chromatogram, followed by gradient elution of late eluting peaks. We therefore optimized the mobile phase to effect an isocratic separation of the angiotensins followed by gradient elution of the larger endothelin peptides to decrease retention time within the same HPLC run.

2.4.2. HPLC equipment

The analytical column was a C_{18} reverse phase (150×3.9 mm I.D.) with 5 μm diameter silica gel having a pore size of 100 Angstrom, void volume of 1.5 ml (DeltaPak, Waters, Mississauga, ON, Canada). A guard column of similar packing material was used. The flow-rate was 1.2 ml/min at ambient temperature. Eluate from the column was monitored at a wavelength of 232 nm; AUFS of 0.001 and coarse zero setting of −0.3 AU using a variable wavelength LC spectrophotometer (Model 481, Waters). The mobile phase delivery system was a Model 600E (Waters). A validated two-injector system was employed to minimize HPLC-derived shadowing. Test samples were applied to the HPLC column with a U6K injector (Waters). For standard calibration purposes a Model 700 WISP Autosampler (Waters) was used. ANG II and ET-1 fractions from samples were collected with a fraction collector (Gilson FC203, Mandel Scientific, Guelph, ON, Canada).

2.4.3. Isocratic separation of angiotensins

2.4.3.1. Preliminary separation assessment

Within the gradient time delay of our HPLC system, an isocratic elution of angiotensins was attempted. In order to develop a separation with reasonable peak spacing and shape we tried different blends of mobile phase, keeping other variables constant. This provided information, which was used to optimize the final mobile phase buffer and to assess possible mechanistic mode of separation. These preliminary mobile phases were (a) 20% CH_3CN in water, (b) 20% CH_3CN in 4 m*M* TEAF, (c) 20% CH_3CN in 4 m*M* HCOOH and (d) 20% CH_3CN in a buffer of 4 m*M* TEAF with 4 m*M* HCOOH. The angiotensins were ANG I, *des* Asp^1 ANG I, ANG II, ANG III, ANG III Val^5, ANG IV, and ANG II (4–8).

2.4.3.2. Effects of TEAF and pH on the separation of angiotensins

Information from the preceding chromatograms (Section 2.4.3.1) was utilized to optimize the separation of angiotensins. Capacity factors of the angiotensins were assessed at TEAF concentrations of 20, 12, 8 and 4 m*M* at constant formic acid concentrations of 52.5 m*M* with 20% CH_3CN. Capacity factors of angiotensins were also assessed at pH 3.94, 3.25, 3.05 and 2.97 at a constant TEAF concentration of 4 m*M* with 20% CH_3CN.

2.4.3.3. Optimized isocratic separation of angiotensins

Optimized mobile phase was 20% CH_3CN in a buffer of 4 m*M* TEAF with 30 m*M* HCOOH in the isocratic separation of angiotensins, ANG I, *des* Asp^1 ANG I, ANG II, ANG III, ANG III Val^5, ANG IV, and ANG II(4–8).

2.5. Separation of angiotensins and endothelins simultaneously

A gradient ion-pair reverse phase HPLC separation of angiotensins, ANG I, ANG II, ANG III, ANG IV and ANG II(4–8), and endothelins, ET-1, ET-2, ET-3 and bET-1 in the same HPLC run was developed. Separation was achieved with a binary gradient of TEAF, HCOOH and CH_3CN. Buffer A and B consisted of 18 and 80% CH_3CN, respectively, in 4.0 m*M* TEAF with 30 m*M* HCOOH. The flow-rate was 1.2 ml/min. A linear gradient was employed, increasing buffer B from 1 to 22% over 15 min with maintenance at the final condition until the last peak eluted.

2.6. HPLC separation and collection of ANG II and ET-1 fractions

Retention times of standard ANG II and ET-1 were used to program a fraction collector to collect ANG II and ET-1 fractions from test samples. After calibration with angiotensin and endothelin standards, the HPLC system was flushed with mobile phase buffer B and a second injector was engaged for the purpose of test sample application. Collected ANG II or ET-1 fractions from samples were dried and stored at −20°C until RIA quantification.

2.7. Radioimmunoassay quantification of collected ANG II and ET-1 fractions

2.7.1. ANG II fraction

Dried ANG II fractions were reconstituted with 0.1 *M* Tris buffer, pH 7.4 and quantified using an ANG II RIA kit (Euro-diagnostica, Apeldoom, The Netherlands). The ANG II antiserum cross-reacted with ANG II, ANG IIVal^5, and ANG III by 100%, and with ANG I by 0.1%. Unknowns were determined from a RIA standard curve that was generated from ANG II concentration range of 1.0 to 64.0 fmol/tube. The standard curve was generated in parallel with test samples. ANG II antiserum was incubated with reconstituted ANG II fractions for 6 h at 4°C. Competitive binding of ^{125}I-ANG II was achieved during a subsequent incubation for 18 h at 4°C. Goat anti-rabbit gamma-globulin bound on solid-phase was used to separate bound from unbound tracer and ANG II. The supernatant was aspirated from the residue after centrifugation at 2000 *g* for 15 min. The residue was then counted for 1 min in a gamma counter (Gamma 5500 counter, Beckman, Mississauga, ON, Canada). Bound relative to maximum binding percentages ($\%B/B_{max}$) versus ANG II concentrations were generated, from which amounts of peptides were determined.

2.7.2. ET-1 fraction

Dried ET-1 fractions were reconstituted with assay buffer (Amersham, Oakville, ON, Canada). Unknowns were determined from a RIA standard curve of ET-1 that was generated from 0.5 to 64.0 fmol/tube. The standard curve was generated in parallel with test sample fractions. A primary rabbit anti-ET-1 serum was added to reconstituted samples and incubated for 16 h at 4°C. Competitive binding of tracer, ^{125}I-ET-1, was achieved with an incubation period of 24 h at 4°C. A second antibody, donkey anti-ET-1 rabbit serum, attached to magnetizable polymer beads was used to separate the primary ET-1 antibody from solution. The solution was decanted and the remaining pellet was counted in a gamma counter (Beckman) for 1 min. Bound relative to maximum binding percentages ($\%B/B_{max}$) versus ET-1 concentrations were generated, from which test samples were determined. The cross-reactivity of the

rabbit anti-ET-1 serum was 100, 204, and 37.9% for ET-1, ET-2 and bET-1, respectively.

2.8. Analytical validation of ANG II and ET-1 measurement

Precision and recovery of ANG II and ET-1 were evaluated for the complete method from a homogeneous sample pool of canine myocardium. Precision was assessed by 5–8 repeated measurements. Recovery was estimated separately with a standard addition of ANG II (50 fmol/g tissue) or ET-1 (91 fmol/g tissue). The mean difference of determined ANG II or ET-1 from the standard addition samples and the samples without added ANG II or ET-1 was expressed relative to the amount of ANG II or ET-1 added, respectively.

2.9. Measurement of ANG II and ET-1 from canine myocardium

The validated HPLC–RIA method was applied to the measurement of ANG II and ET-1 from heart tissues of seven normal mongrel dogs. Free wall samples from the right atrium (RA), right ventricle (RV), left atrium (LA), and left ventricle (LV) were assayed.

3. Results

3.1. HPLC method development: preliminary assessment of angiotensin isocratic separation

Chromatograms of angiotensins, ANG I, *des* Asp^1 ANG I, ANG II, ANG III, ANG III Val^5, ANG IV, and ANG II (4–8), under various isocratic mobile phase conditions are shown in Fig. 1a 20% CH_3CN in water, (b) 20% CH_3CN in 4 m*M* TEAF, (c) 20% CH_3CN in 4 m*M* HCOOH, (d) 20% CH_3CN in 4 m*M* TEAF with 4 m*M* HCOOH. Other conditions, column, flow-rate, injection volumes, were the same for each chromatogram. Extensive peak tailing or unsymmetrical peaks were observed with a mobile phase of 20% CH_3CN in water. A mobile phase with TEAF eliminated peak tailing, however angiotensins co-eluted. Formic acid improved peak selectivity, but at the expense of peak tailing. A combination of TEAF and formic acid with acetonitrile produced acceptable peak shape and spacing of the angiotensins. However, ANG IV and *des* Asp^1 ANG I co-eluted, but were subsequently resolved (see Section 3.1.2).

3.1.1. Effects of TEAF and pH on angiotensin separation

Capacity factors, k', versus TEAF concentrations and varying pHs are shown in Fig. 2a and b, respectively. At a TEAF concentration of 4 m*M* the angiotensins were separated with baseline resolution at k' of less than 4.5. Angiotensins were separated with a minimum k' of 4.06 at pH 3.05.

3.1.2. Optimized isocratic separation of angiotensins

Fig. 3 shows the chromatogram of the isocratic separation of angiotensins, ANG III Val^5, ANG II (4–8), ANG III, ANG II, *des* Asp^1 ANG I, ANG IV, and ANG I. All peaks were resolved within 8 min, including ANG IV and *des* Asp^1ANG I.

3.1.3. Optimized separation of both angiotensins and endothelins

TEAF, formic acid and acetonitrile concentrations were subsequently optimized as in buffer A and B under the influence of a linear gradient to produce a separation of angiotensins and endothelins within 25 min as shown in Fig. 4a. Table 2 is a summary of angiotensin and endothelin peak characteristics.

3.2. HPLC separation and collection of tissue ANG II and ET-1

Fig. 4b shows a HPLC profile of tissue extract at 232 nm and collection windows for ANG II and ET-1. Collection windows were based on retention times for ANG II and ET-1. Retention times mean$\pm$SD of ANG II and ET-1 on different days with different batches of mobile phase were 7.26 ± 0.06 and 22.45 ± 0.22 min ($N=4$), respectively.

3.3. RIA quantitation of collected ANG II and ET-1 fractions

Fig. 5 shows RIA standard curves of (a) ANG II and (b) ET-1, which were used to determine test

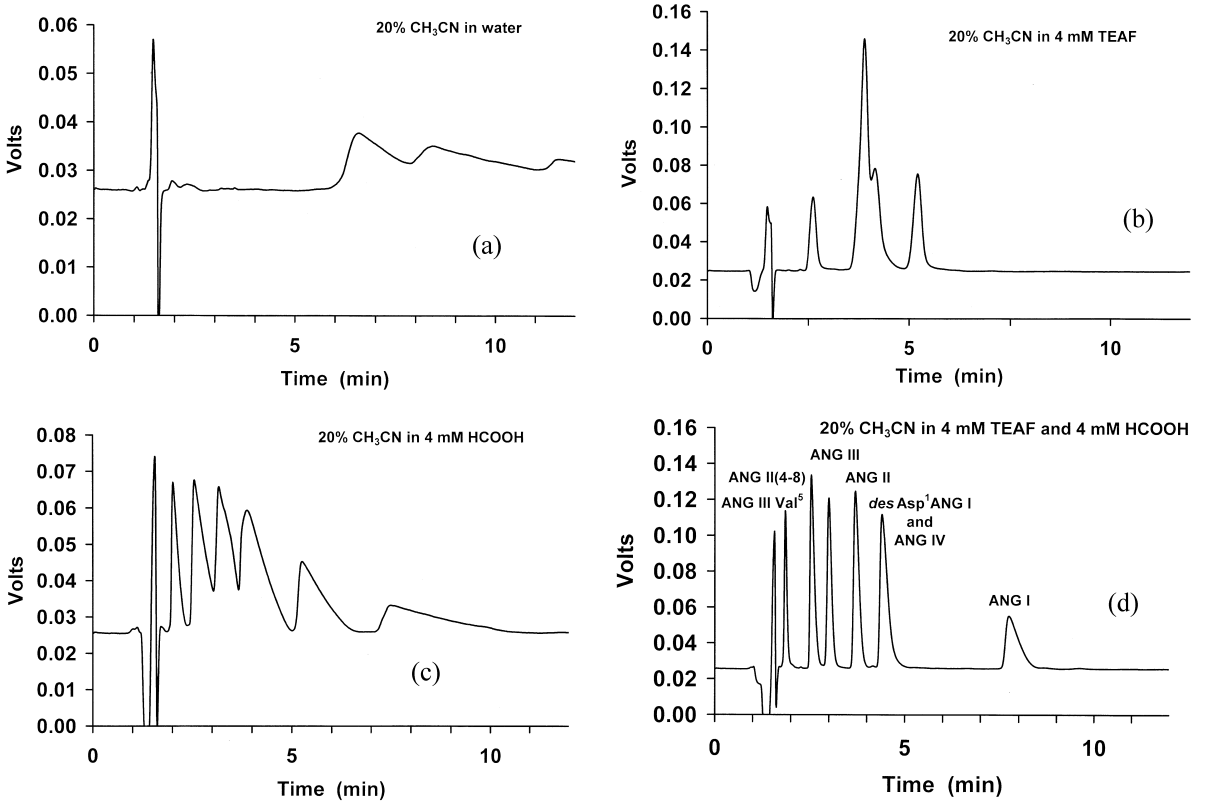


Fig. 1. Chromagograms of angiotensins, ANG I, *des* Asp[1] ANG I, ANG II, ANG III, ANG III Val[5], ANG IV, and ANG II (4–8), under different isocratic mobile phase conditions: C_{18} column; flow=1.2 ml/min; λ=232 nm; (a) 20% CH_3CN in water, (b) 20% CH_3CN in 4 m*M* TEAF, pH 6.0, (c) 20% CH_3CN in 4 m*M* formic acid, pH 3.43 and (d) 20% CH_3CN in 4 m*M* TEAF with 4 m*M* formic acid buffer, pH 3.94.

sample levels. The effective dose (ED) at ED_{80}, ED_{50}, and ED_{20} mean±SD of the ANG II curve were 3.9±1.6, 10.1±1.2, 34.8±2.8 fmol/tube (N=4) and for the ET-1 standard curve were 2.6±0.4, 8.8±0.4, 35.0±3.6 fmol/tube (N=4), respectively. Percentage B/B_{max} were within standard curve ranges from unknown samples. The detection limit of ANG II and ET-1 were 0.56 and 1.03 fmol/tube, respectively.

3.4. Analytical validation of ANG II and ET-1 measurements

Precisions (coefficient of variation) for the measurement of ANG II and ET-1 concentrations from the myocardium were 14.0% (N=5) and 14.8% (N=8), respectively. Myocardium recovery rates for ANG II and ET-1 were 42.2% and 62.1%, respectively.

3.5. Canine myocardium ANG II and ET-1 concentrations

Table 3 shows a summary of the raw data and mean±SD of myocardial ANG II and ET-1 concentrations in fmol/g from seven normal dogs in the RA, RV, LA, and LV.

4. Discussion

The measurement of ANG II and ET-1 from tissue requires a specific and a sensitive method, as provided by HPLC in combination with RIA. RIA is a

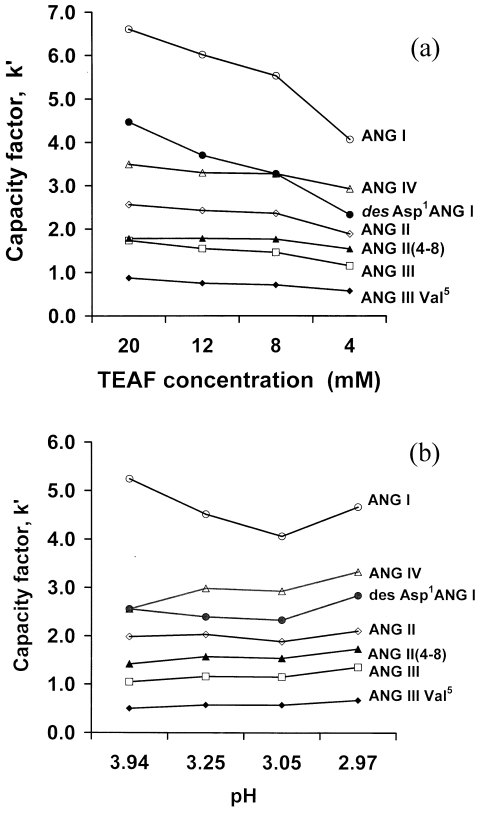


Fig. 2. Capacity factor, k', vs. various mobile phase (a) TEAF concentrations in 52.5 m*M* HCOOH with 20% CH_3CN and (b) pH of 4 m*M* TEAF with 20% CH_3CN buffer. Other experimental conditions were the same, C_{18} column, flow=1.2 ml/min, λ=232 nm.

binding technique, which relies on interaction between antigen, ANG II or ET-1, and its binder, ANG II or ET-1 antibody, respectively. Antibody-antigen interactions in RIA are competitively challenged by antigen labelled (tracer) with a gamma radiation emitting isotope, such as ^{125}iodine, allowing for low detection of endogenous antigens. The key limitation of RIA is that antibodies to ANG II or ET-1 have limited selectivity. These antibodies do not differentiate between individual peptide members within each family. This non-specificity of antibodies is especially prevalent with small antigens as in the angiotensin and the endothelin peptide families. HPLC is capable of selectively separate similar peptide members within each group. However, conventional HPLC detectors are not sensitive enough to

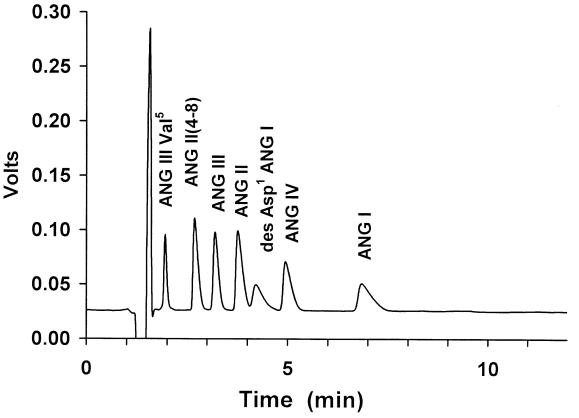


Fig. 3. Isocratic separation of angiotensins, mobile phase consisted of 20% CH_3CN in a buffer of 4 m*M* TEAF and 30 m*M* HCOOH, C_{18} column, flow=1.2 ml/min, λ=232 nm. Note resolution of *des* Asp^1 ANG I, and ANG IV.

detect low endogenous ANG II and ET-1 amounts. Therefore, the utilization of HPLC with RIA can provide the necessary specificity, as well as, sensitivity, respectively, in the measurement of a wide range of endogenous ANG II or ET-1 concentrations.

4.1. HPLC method development: separation of angiotensins and endothelins

We developed an ion-pair reversed-phase HPLC method to separate angiotensin and endothelin peptides simultaneously. Reverse-phase chromatography separation is dependent on the partition of angiotensins and endothelins between two distinct phases, an aqueous mobile phase and a hydrophobic stationary phase. Although these peptides are soluble in aqueous solutions, they have nonpolar amino acid residues or hydrophobic regions within their structures which can interact with the hydrophobic stationary phase. The nonpolar amino acid residues of angiotensins are valine, tyrosine, isoleucine, proline, phenylalanine and leucine. As the hydrophobicity of the peptide increases, so does the time it resides on the column. Changing the ingredients of the mobile phase can shift the distribution or partition coefficient between stationary and mobile phase resulting in changes in migration rates of angiotensins and endothelins. In addition to nonpolar amino acid residues, angiotensins and endothelins have amino

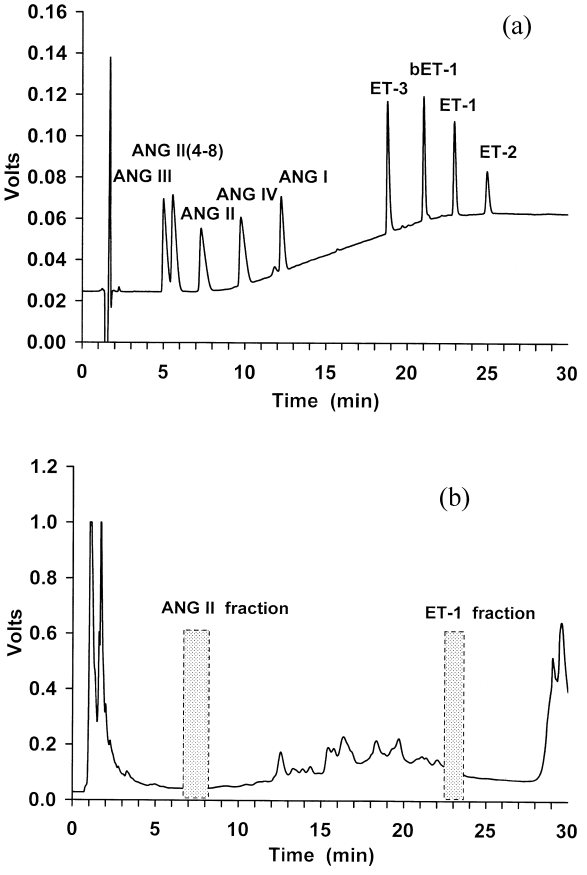


Fig. 4. (a) Chromatogram of optimized separation of angiotensins and endothelins. Conditions: reversed-phase C_{18} column, flow= 1.2 ml/min, detection λ=232 nm, linear gradient mobile phase of buffer B of 1–22% for 15 min. Buffer B and A consisted of 80 and 18% CH_3CN, respectively, in 4 m*M* TEAF with 30 m*M* HCOOH. (b) Chromatogram of tissue extract showing ANG II and ET-1 collection windows. HPLC conditions were the same as in (a).

acid residues with functional side groups. These functional groups, such as the carboxylate anion, primary and secondary amine cations, as well as terminal carboxylate and amine ions of peptides, can influence separation quality. Functional groups ionization is modulated by mobile phase pH and/or with mobile phase additives, such as ion-pairing reagents. For example, mobile phase pH modification allows for protonation of carboxylate anions which increases the hydrophobicity of such peptides. Ion-pairing reagents of opposite charge also enhance the hydrophobicity of these peptides by neutralizing their charge [30]. A second function of mobile phase additives is to improve peak symmetry by decreasing peak tailing. The strategy followed in the angiotensin and endothelin HPLC method development is to manipulate their migration rates to produce a chromatogram with symmetrical peak shape, maximize selectivity and resolution between peaks and to have elution occur in a relatively short time period.

The mobile phase was optimized to achieve an isocratic separation of angiotensins within the gradient delay time of our HPLC system. We have used acetonitrile, triethylammonium formate (TEAF) and pH adjustment to develop a separation of the angiotensins. Under reversed-phase conditions, separation of angiotensins was inefficient as shown in the chromatogram of Fig. 1a. Angiotensins have a high affinity for the stationary reversed-phase column packing versus a mobile phase of water and organic modifier of 20% acetonitrile. This is evident by the extensive tailing of peaks in the chromatogram, which indicate both hydrophobic and silanophilic interactions [31]. Silanophilic interaction is a mechanism of peak tailing [31] that contributes to asymmetric angiotensin peaks. Although hydrophobic C_{18} material is covalently bonded to silica gel and then further end-capped to prevent the interaction of silanol groups and eluites, there still exists free silanol groups in reversed-phase stationary materials. These free silanol groups have net dipole moments of small negative charges, which attract positive charged amine cations of the angiotensins. Such silanophilic interactions result in asymmetric peak shapes in reversed-phase chromatography, as exemplified by the peaks from angiotensins.

Mobile phase with TEAF improved peak symmetry and reduced retention time as shown in the chromatogram of angiotensins of Fig. 1b. Ion-pair, reversed-phase chromatography employs two groups of ion-pairing reagents with different mechanisms of reducing peak tailing, the cationic, such as ammonium acetate and TEAF, and the anionic, such as alkylsulphonates and trifloroacetic acid [30,32]. The triethylammonium (TEA) ion has a positive charge, which binds to free silanol groups of the stationary phase, preventing interaction of positive charged groups of the angiotensins. Amines added to mobile phase to mask the effects of silanol groups are effective in reducing the dependence of retention on

Table 2
Chromatographic peak parameters of angiotensins and endothelins

	T[a] (min)	W[b] (min)	k'[c]	α[d]	R[e]	N[f]	$T_{0.05h}$[g]
ANG III	5.0	0.48	3.03			1708	2.10
				1.16	1.12		
ANG II(4–8)	5.6	0.55	3.50			1695	1.93
				1.40	2.86		
ANG II	7.3	0.66	4.90			1963	1.91
				1.40	3.64		
ANG IV	9.7	0.67	6.85			3374	1.78
				1.29	4.58		
ANG I	12.2	0.42	8.86			13 438	1.34
				1.60	18.14		
ET-3	18.7	0.30	14.15			62 700	1.38
				1.13	8.26		
bET-1	21.0	0.24	15.94			122 617	1.19
				1.10	7.15		
ET-1	22.9	0.29	17.46			102 490	1.29
				1.10	6.42		
ET-2	24.9	0.35	19.10			81 176	1.37

[a] T=Retention time.
[b] W=Peak width.
[c] k'=Capacity factor=$(t - t_0)/t_0$.
[d] α=Selectivity=k'_2/k'_1.
[e] R=Resolution=$2(t_2 - t_1)/(W_2 + W_1)$.
[f] N=Column efficiency=$16(t_1/W_1)^2$.
[g] $T_{0.05h}$=Tailing factor at 0.05 peak height=$W_{0.05h}/2f$. Column dead-time, t_0=1.24 min.

sample size, improving column efficiency, sample recovery, and normalizing the retention behaviour of nitrogen-bearing eluites [31]. The competing base effects of TEA for silanol groups reduce peak tailing and improve the peak shape of the angiotensins. Other laboratories have used anionic ion-pairing reagents in the isocratic separation of angiotensins with improved peak symmetry, however, at the cost of longer retention times [33]. Alkylsulphonates prevent silanophilic interactions as well by binding to cations of angiotensins, but this type of ion-pairing reagent made the angiotensins more hydrophobic and elution occurred over a longer time period. TEAF improved angiotensin peak symmetry by reduction of peak tailing, however, with diminished peak selectivity or extensive peak co-elution.

The suppression of carboxylate anions of angiotensins minimally improved peak symmetry, but increased peak selectivity. Fig. 1c shows a chromatogram of angiotensins from a mobile phase with formic acid but with no TEAF. Overall retention was longer, however the peaks were more unsymmetrical than with TEAF alone in the mobile phase, cf. Fig. 1b. The longer retention was a result of carboxylate ion suppression at a lower pH, making the angiotensins more hydrophobic and therefore more retentive on the stationary phase. Angiotensin peak tailing at lower pH mobile phase was not as severe as at neutral pH (see Fig. 1a). At lower mobile phase pH more protons are available to bind to silanol groups with small negative charge. This effectively reduced silanophlic interaction with cations of angiotensins, resulting in a minimal improvement of peak symmetry. This modulation of peak tailing by pH induced silanol charge suppression, however, was not sufficient, an ion-pairing reagent such as TEAF, was required.

A blend of mobile phase with a combination of TEAF and formic acid enhanced peak symmetry and selectivity of the angiotensins, as shown in the chromatogram of Fig. 1d. The majority of the angiotensin peaks attained baseline separation, that is resolution of 1.5 and greater, except angiotensin hexapeptide ANG IV and *des* Asp^1 ANG I. These

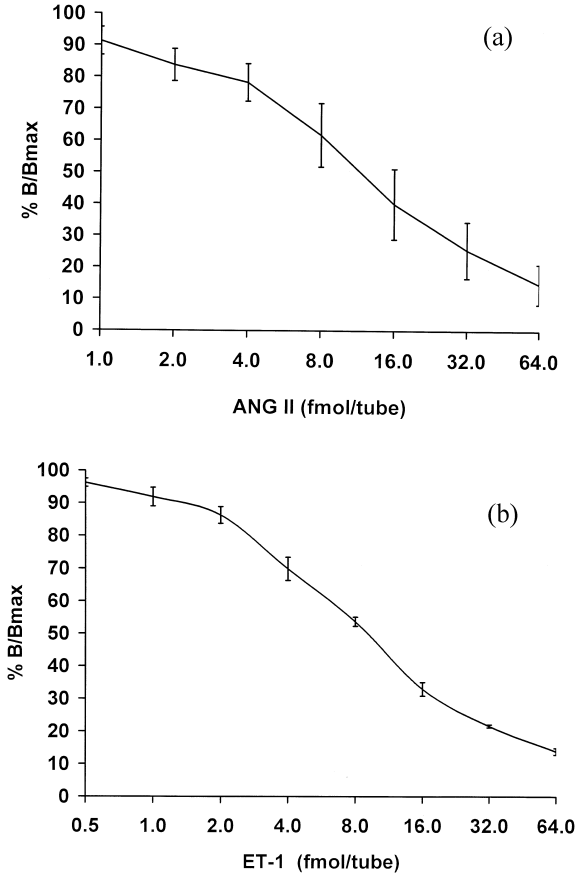


Fig. 5. RIA standard curves (a) ANG II and (b) ET-1. Plot of mean±SD of bound relative to maximum binding percentages ($\%B/B_{max}$) versus standard ANG II or ET-1 in fmol/tube ($N=4$), respectively. Unknowns were within the standard curve ranges.

co-eluting peaks were resolved by studying the effects of mobile phase pH variation and TEAF concentrations on capacity factors.

Mobile phase with TEAF and lower pH was effective in improving peak shape and reducing retention time. TEA acts as a mobile phase ion-pairing reagent, which increase or decrease retention time of eluites [34]. Within the TEAF concentrations in the range of 4–20 m*M* (pH 3.1–3.3), the capacity factors of angiotensins decreased as TEAF concentration decreased, as shown in Fig. 2a. This is clearly observed with ANG I. At higher TEAF concentrations there are more TEA ions available to pair with the ionized caboxylate group(s) making the peptides more hydrophobic. Repulsion of cationic groups of angiotensins by TEA seems to be constant in the experimental TEAF concentration range. Mobile phase pH variation between 3.94 and 2.97 produced a bi-phasic change in retention time, however, as demonstrated by ANG I in Fig. 2b. Minimum capacity factor, k', of 4.06 occurred at pH 3.05. As the pH increased from 3.05, the retention time increased. This increase in retention time is attributed to the presence of more carboxylate anions of the angiotensins at higher pH, which can bind to TEA. The binding of TEA to carboxylate anions made the angiotensins more hydrophobic, resulting in higher k'. As the pH decreased further to below 3.05, k' increased again. A different mechanism, other than ion-pairing, is responsible for this increase in retention time. Lower pH resulted in the suppres-

Table 3
Normal canine myocardium ANG II and ET-1 concentrations in fmol/g ($N=7$)

	ANG II				ET-1			
	RA[a]	RV[b]	LA[c]	LV[d]	RA[a]	RV[b]	LA[c]	LV[d]
	39.6	11.7	23.3	13.5	72.0	61.6	64.6	42.2
	25.0	17.5	17.6	21.1	42.1	55.2	96.0	134.2
	45.0	52.5	28.3	35.7	23.4	63.1	45.2	70.6
	38.6	5.8	26.0	21.5	55.0	35.7	63.7	35.1
	49.9	50.9	34.9	15.0	27.5	18.6	43.7	33.2
	50.6	43.2	40.9	25.8	32.7	30.7	38.5	36.2
	24.7	12.2	43.9	17.3	50.9	29.2	35.7	45.4
Mean±SD	39±11	28±21	31±11	21±8	43±16	42±19	55±21	57±34

[a] RA=Right atrium.
[b] RV=Right ventricle.
[c] LA=Left atrium.
[d] LV=Left ventricle.

sion of carboxylate anions of angiotensins, producing longer retention times. From these experiments, the optimum separation was achieved at a pH of 3.05 to 3.25 with 4 m*M* TEAF concentration buffer, as shown in the isocratic separation of angiotensins of Fig. 3.

Gradient elution of the endothelin family occurred later in the combined separation, with the last peak having a retention time of just less than 25 min. The gradient was optimized and the final separation of both families of immuno-reactive peptides, ANG III, ANG II(4–8), ANG II, ANG IV, ANG I, ET-3, bET-1, ET-1, and ET-2 is shown in the chromatogram of Fig. 4. Capacity factors, k', ranged from 3 to 19. An upward drift in the baseline resulted from increasing acetonitrile concentration of the mobile phase by the gradient. Peak efficiencies of the endothelin family, under the influence of the mobile phase gradient, were superior to those for the isocratically separated angiotensin family. Differences in peak efficiency are attributable to the influence of the gradient on endothelins. The gradient increased the acetonitrile concentration of the mobile phase over time, resulting in an increasingly hydrophobic mobile phase. Under such conditions endothelins change their distribution between the stationary and the mobile phase, preferring the mobile phase. As a result endothelins spend less time, as reflected in narrower peak widths, on the column at appropriate mobile phase acetonitrile concentrations.

4.2. Application of HPLC separation and RIA quantification of angiotensins and endothelins in myocardium extracts

The HPLC separation of angiotensins and endothelins was applied to canine myocardium extracts. Although we have successfully separated immuno-reactive species within each peptide family, it is conceivable that other endogenous substances may have co-eluted with peaks from these tissue extracts. However, ANG II and ET-1 antibodies are selective enough not to cross-react with co-eluting substances other than angiotensins and endothelins, respectively. Retention times of standard angiotensins and endothelins were used to calibrate a fraction collector to collect ANG II and ET-1 peaks from myocardium extracts in separate tubes. The retention time calibrations were reproducible for both ANG II and ET-1 peaks, with less than 1% variation between days. An additional benefit of using TEAF as an ion-pairing reagent of the mobile phase is its volatility, which allows for easy drying of peak fractions. A problem with using standards for calibration purposes is their carryover to test samples resulting in false positive results. We addressed this problem by using a validated two-injector HPLC system, one injector reserved for standards with a second for test sample application. The minimization of carryover between sample application, the HPLC and RIA selectivity improved the accuracy of ANG II and ET-1 measurement.

Overall analytical recoveries of ANG II and ET-1 by standard addition were 42.2 and 62.1% from canine myocardium, respectively. Adjustment of pH in the angiotensin extraction from myocardium was tedious, especially when amounts of tissue varied. This may have contributed to a relatively low recovery of ANG II from the myocardium. Solid-phase extraction procedures, however, resulted in recoveries of ^{125}I-ANG II and ^{125}I-ET-1 of over 80% (data not shown). The gradient separation of ET-1 was more efficient, *N*, than the isocratic separation of ANG II, $N=102\,490$ and 1963, respectively. ANG II peak tailing is an indication that ANG II has a longer residence time on the column stationary phase, which may have contributed to a lower recovery rate. Presented measured tissue ANG II and ET-1 concentrations were not corrected with these recovery rates.

Reproducibility of ANG II and ET-1 was assessed by repeated measurements from a homogeneous sample pool. The precision of ANG II and ET-1 were 14.0 and 14.8%, respectively. The detection limits were 2.8 and 5.0 fmol/g for ANG II and ET-1, respectively. Unknowns were within the ANG II and ET-1 standard curves shown in Fig. 5a and b, respectively.

5. Conclusion

In conclusion, the HPLC separation of ANG II and ET-1 from their respective immuno-reactive family members resulted in specific measurements by RIA. The accuracy of our measurements is

improved by the minimization of HPLC-derived shadowing of our HPLC system and the enhanced RIA selectivity provided by HPLC isolation of immuno-reactive species. Individual members of both angiotensin and endothelin peptide families, ANG III, ANG II(4–8), ANG II, ANG IV, ANG I, ET-3, bET-1, ET-1 and ET-2, were separated simultaneously within 25 min. The gradient separation of endothelins was superior to the isocratic separation of angiotensins. Normal canine myocardium, right atrium, right ventricle, left atrium and left ventricle, ANG II concentrations were 39±11, 28±21, 31±11 and 21±8, and ET-1 concentrations were 43±16, 42±19, 55±21 and 57±34 fmol/g, respectively. The simultaneous HPLC separation of the angiotensins and endothelins may be applied to the selective measurement of other immuno-reactive members of those families.

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