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Specific and non-specific measurements of tissue angiotensin II cascade members

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Abstract

Although angiotensin II (ANG II) has been the focal regulatory peptide of the renin–angiotensin system, its proteolytic fragments have recently been demonstrated to have biological effects. Conventional measurement of angiotensins involves radioimmunoassay (RIA), which is a sensitive binding technique capable of measuring low physiological concentrations. However, ANG II antibody cross-reacts with ANG II and its fragments (ANG II cascade), rendering RIA measurement alone to be a non-specific measure of immunoreactive ANG II (ir-ANG II). On the other hand, high-performance liquid chromatography (HPLC) is capable of separating immunoreactive ANG II cascade members, but may not be sensitive enough to detect these low peptide concentrations often present in biological samples. Consequently, a reverse-phase HPLC method, with triethylammoniun formate as an ion-pair reagent, was developed to separate ANG II and its fragments, ANG III, ANG IV and ANG V. This HPLC separation was applied to extracts from normal canine hearts and ANG II cascade immunoreactive fractions were collected. Collected fractions were quantified by RIA, with the use of separate standard curves. The isocratic HPLC separation of ANG II, ANG III, ANG IV and ANG V was achieved in less than 5 min with adjacent peaks having baseline resolution. Measured cardiac left ventricle ANG III, ANG IV and ANG V concentrations (mean $\pm$ SD) were 5.3 ± 2.2, 4.0 ± 1.0 and 3.1 ± 1.0 fmol/g ($n = 9$), respectively. There was a significant difference ($P = 0.003$, $n = 9$) between left ventricular immunoreactive ANG II and 'true' ANG II, corrected for recovery rates of 86.2 ± 22.5 and 53.5 ± 16.2 fmol/g, respectively. We conclude that the combination of HPLC with RIA ensures the specific measurement of the ANG II cascade family members while non-chromatographic processing of tissue renders ANG II measurement non-specific. In addition, the use of triethylammonium formate as mobile phase additive is superior in the HPLC separation of the angiotensins.

Keywords: Angiotensins; Immunoreactive angiotensins; Tissue extraction; Solid-phase extraction; Triethylammonium formate; Ion-pair reverse phase high-performance liquid chromatography; Radioimmunoassay

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1. Introduction

Angiotensin II (ANG II) cascade, peptides derived from the sequential proteolytic cleavage of ANG II, is a component of the renin–angiotensin system (RAS). These ANG II metabolites are more complex functionally than was previously thought. The RAS plays an integral role in maintaining circulation homeostasis and is observed in the circulation and in many organs including the heart [1–3]. ANG II, produced from two sequential enzymatic steps with the action of renin on angiotensinogen to angiotensin I (ANG I) followed by the action of angiotensin converting enzyme (ACE), has been the focal regulatory hormone of this peptide family in normal physiology and pathophysiology. Indeed, it is well established that ACE inhibitors and ANG II receptor antagonists are useful therapeutic agents in diseases in which ANG II is elevated, such as in hypertension and heart failure [4,5]. ANG II metabolites, once viewed as inactive fragments with no function, however, have unique biological effects [6].

Besides ANG II, ANG I produces angiotensin-(1–7) (ANG-(1–7)). ANG-(1–7) has vasodilatory and antihypertensive actions which may counterbalance some of ANG II's effects [7]. ANG II is transformed to angiotensin III (ANG III) by aminopeptidase A and the action of aminopeptidase B on ANG III produces angiotensin IV (ANG IV), which in turn is transformed to angiotensin V (ANG V) [8]. Two identified cell surface receptors, AT_1 and AT_2, mediate ANG II physiological actions [9,10]. However, a different receptor, AT_4, mediates the effects of ANG IV [11]. It is believed that ANG III action is mediated via the AT_1 receptor; while ANG III pressor response is weaker than ANG II, its depressor action is stronger [12]. No unique receptors to date have been identified with ANG V or ANG-(1–7). ANG II has vasoconstrictive and trophic effect [13]. On the other hand, ANG IV has antitrophic effect [14] and has shown to increase blood flow [15]. These proteolytic products of ANG II have biological effects, which in some cases are opposite to that of ANG II. It is therefore, desirable to measure these ANG II fragments in addition to ANG II, the ANG II cascade.

The measurement of physiological concentrations of endogenous substances, such as angiotensins, utilizes radioimmunoassay (RIA). RIA is a sensitive technique, which relies on the binding of the peptide and its antibody. Antibodies however, are capable of cross-reacting with peptide members within a family. ANG II antibody is non-specific to the binding of ANG II, it binds not only to ANG II, but also to ANG III, ANG IV and ANG V. This non-specificity of ANG II antibody can be of advantage in the specific measurement of individual ANG II cascade members, provided that each cascade members is isolated from each other prior to RIA quantification. An efficient method of peptide separation can be achieved by reverse phase high-performance liquid chromatography (HPLC) [16]. HPLC is capable of distinguishing small structural peptide differences within a family; however, conventional HPLC detectors are unable to detect low physiological amounts of ANG II cascade members. The combination of these two techniques, HPLC and RIA, can provide the required specificity and sensitivity, respectively, in the measurement of physiological concentrations of ANG II and its fragments.

In addition to having hormonal function, the RAS may have paracrine and/or autocrine properties in the myocardium. We have previously reported myocardial ANG II concentrations in the dog [23] and now sought to report ANG III, ANG IV and ANG V levels measured by HPLC and RIA. We also measured myocardial immunoreactive ANG II (ir-ANG II) concentrations, a concurrent measurement of all cross-reactive ANG II cascade members by RIA alone, without chromatographic processing.

2. Experimental

2.1. Peptides and chemicals

ANG I (ANG I(1–10)), ANG II (ANG I(1–8)), ANG III (ANG II(2–8)), (Bachem, Philadelphia, PA); ANG IV (ANGII(3–8)), ANG V

(ANG II(4–8)) (Bachem California, Torrance, CA); formic acid (HCOOH) (Sigma, St Louis, MO). HPLC grade solvents: methanol (CH_3OH) and acetonitrile (CH_3CN) (EM Science, Toronto, ON, Canada); triethylammonium formate ($(C_2H_5)_3NCOOH$, TEAF) (Fluka, Ronkonkoma, NY). 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 before the commencement of the experiments.

2.3. Acidified ethanol tissue extraction and solid-phase extraction

Frozen tissue samples were thawed at 4°C, cleaned, and 0.3110 ± 0.0068 g wet weight was homogenized with a Polytron (Brinkmann Instruments, Streetsville, ON, Canada) in 20 volumes of ice-cooled 0.18 M HCl:ethanol (1:3 v/v) [17]. 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, obtained after centrifugation, was decanted and its pH adjusted between 5.5 and 6.0 with 1 M NaOH and then kept on ice for 1 h. The supernatant was further centrifuged at 2200*g* (OmnifugeRT, Baxter, Mississauga, ON, Canada) for 10 min at 4°C. Adjustments to pH 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. Finally, the supernatant was decanted and evaporated in a vacuum concentrator (Savant, Farmingdale, NY) and was stored at −20°C.

Solid-phase extraction (SPE) was performed on reconstituted tissue extract with the use of Sep Pak C_{18} columns (Waters, Mississauga, ON, Canada). Columns were conditioned with 6 ml each of methanol and deionized water. The dried supernatant was reconstituted with 2×500 ul of 1 M formic acid and applied to a pre-conditioned Sep Pak. The loaded Sep Pak column was washed with 6 ml of 10% methanol in 1 M formic acid. ANG II and its fragments were eluted with 8 ml of 80% methanol in 1 M formic acid, and the eluent dried in a vacuum concentrator.

2.4. High performance liquid chromatography

An analytical reverse phase C_{18} column (150 × 3.9 mm ID) with 5 μm diameter silica gel having a pore size of 100 Angstrom, void volume of 1.5 ml (DeltaPak, Waters, Mississauga, ON, Canada) and a guard column of similar packing material were 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 [40]. 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, ANG III, ANG IV and ANG V fractions from samples were collected with a fraction collector (Gilson FC203, Mandel Scientific, Guelph, ON, Canada).

Optimized mobile phase, buffer A, of 20% CH_3CN in 4 mM TEAF with 30 mM HCOOH was effective in the isocratic separation of ANG I, ANG II, ANG III, ANG IV and ANG V. Tissue extract from SPE was reconstituted with 100 μl of mobile phase and 95–97 μl of which was injected onto the column. A washout buffer B was used between sample application of 80% CH_3CN in 4 mM TEAF with 30 mM HCOOH.

2.5. HPLC separation and collection of angiotensin fractions

Retention times of angiotensin standards were used to program a fraction collector to collect angiotensin fractions from tissue extracts. Fractions were collected between 2.4–2.8, 2.9–3.2, 3.4–3.9 and 4.5–5.1 min for ANG III, ANG V, ANG II and ANG IV, respectively. After calibration with angiotensin standards, the HPLC system was flushed with mobile phase buffer B and a second injector engaged for the purpose of test sample application. Collected angiotensin fractions from samples were dried and stored at −20°C until RIA quantification.

2.6. Radioimmunoassay quantification of collected angiotensin fractions

Dried ANG II, ANG III, ANG IV and ANG V fractions were reconstituted with 0.1 M TRIS buffer, pH 7.4 and quantified using an ANGII RIA kit (Euro-diagnostica, Apeldoom, The Netherlands). The ANG II antiserum cross-reacted with ANG II and its fragments. Unknown fractions were determined from ANG II, ANG III, ANG IV and ANG V RIA standard curves, generated in parallel with test samples. ANG II antiserum was incubated with reconstituted ANG II, ANG II, ANG IV and ANG V fractions for 6 h at 4°C. Competitive binding of tracer, ^{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. The supernatant was aspirated from the residue after centrifugation at $2000 \times g$ for 15 min. The residue was then counted for 1 min in a gamma counter (Gamma 5500 counter, Beckman, Mississauga, ON, Canada). Unknown amounts were determined from standard curve plots of bound relative to maximum binding percentages ($\%B/B_{max}$) versus ANG II, ANG III, ANG IV, and ANG V concentrations in fmol/tube.

2.7. Measurement of ANG II, ANG III, ANG IV and ANG V from normal canine heart

ANG II, ANG III, ANG IV and ANG V measured in nine left ventricles by the sequential combination of procedures, acidified ethanol tissue extraction, SPE, HPLC and RIA (AETE/SPE/HPLC/RIA). Spiked recovery assessment of the combined procedures was performed using standard ANG II ($n = 5$). Intra-assay precision was assessed by repeated measurements of ANG II ($n = 5$) from a homogenized pool of left ventricle.

2.8. Measurement of immunoreactive ANG II (ir-ANG II) from normal canine heart

Tissue samples (0.1401 ± 0.0178 g wet weight) were extracted using hot acetic acid [18,19]. Pieces of left ventricular myocardium were boiled in 0.1M acetic acid for 10 min and then homogenized. Samples were cooled on ice followed by centrifugation at $2000 \times g$ for 20 min at 4°C. The supernatant was decanted and dried in a vacuum concentrator (Savant). This tissue extract was reconstituted, but it was neither applied to SPE nor to HPLC columns. RIA was used to quantify ir-ANG II with the use of ANG II standard curve. Spiked recovery assessment of this extraction procedure was performed using standard ANG II ($n = 5$). Intra-assay precision was assessed by repeated measurements ($n = 5$) from a homogenized pool of left ventricle. This hot acetic acid tissue extraction followed by RIA (HAATE/RIA) was performed on nine left ventricles.

2.9. Data analysis

Measured concentrations of ANG II, ANG III, ANG IV, ANG V, and ir-ANG II from tissue are expressed as mean ± SD in fmol/g. Differences in tissue weights and volumes are reflected in the final calculated concentration values. Significance of ANG II versus ir-ANG II concentrations, measured by AETE/SPE/HPLC/RIA and HAATE/RIA methods, respectively, was obtained by using Student's t-test of

two-tailed and two-sampled unequal variance distribution.

3. Results

3.1. HPLC separation of angiotensins and collection of fractions

Fig. 1 a and b show chromatograms of angiotensin standards and left ventricular cardiac extract, respectively. Retention times mean ± SD of ANG III, ANG V, ANG II, ANG IV and ANG I were 2.57 ± 0.012, 3.07 ± 0.010, 3.58 ± 0.000, 4.67 ± 0.017 and 6.33 ± 0.029 min ($n = 4$), respectively. Resolution (R) between adjacent peaks, ANG III and ANG V, ANG V and ANG II, ANG II and ANG IV, and ANG IV and ANG I were 2.0, 1.6, 3.1 and 3.4, respectively.

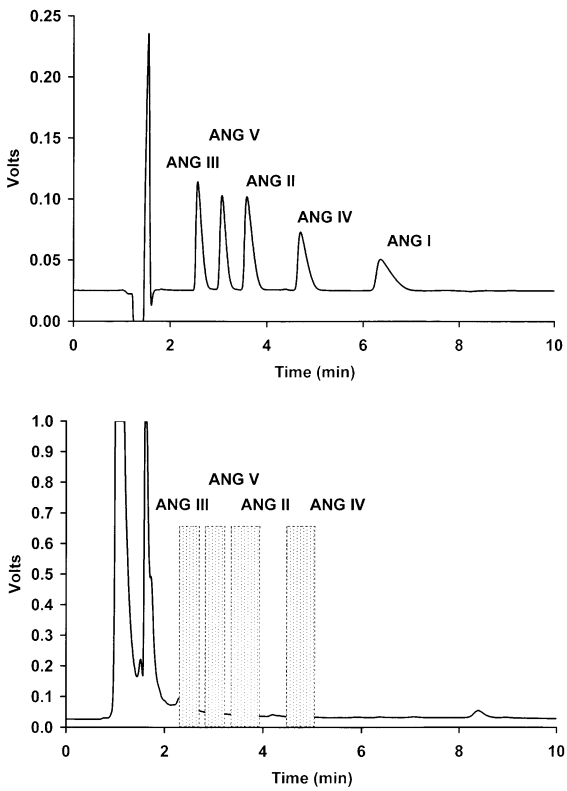

Fig. 1. (a) Chromatogram of the optimized separation of angiotensins. Conditions reverse-phase C_{18} column, flow = 1.2 ml/min, detection $\lambda = 232$ nm, mobile phase of 20% acetonitrile in 4 mM TEAF with 30 mM formic acid; (b) Chromatogram of tissue extract showing ANG III, ANG V, ANG II and ANG IV collection windows. HPLC conditions were the same as in (a).

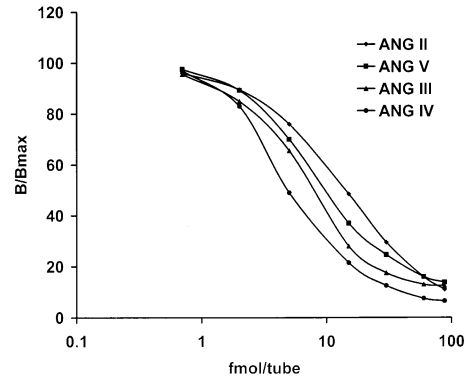

Fig. 2. RIA standard curves of ANG II, ANG III, ANG IV and ANG V, from which unknowns were determined.

3.2. RIA quantification of collected fractions

Fig. 2 shows standard curves of ANG IV, ANG III, ANG V and ANG II having ED_{50} values of 4.8, 6.6, 8.2 and 12.0 fmol/tube, respectively. The ANG II standard curve is least sensitive, while ANG IV is most sensitive. Unknown values were within the standard curve ranges.

Table 1
Normal canine left ventricular cardiac ANG II, ANG III, ANG IV, ANG V in fmol/g, raw data and mean ± SD ($n = 9$) [a]

ANG II[b]	ANG III	ANG IV	ANG V
26.81	1.95	3.35	2.69
13.49	2.97	3.54	2.65
21.12	3.02	3.49	3.63
35.65	7.81	3.21	2.68
24.54	7.09	5.96	2.73
15.02	4.88	4.44	2.59
25.76	6.52	4.12	2.65
17.31	7.96	4.36	2.67
22.62	5.74	4.29	5.49
22.5 ± 6.8	5.3 ± 2.2	4.0 ± 1.0	3.1 ± 1.0

[a] Concentrations determined by acidified ethanol tissue extraction/SPE/HPLC/RIA.

[b] Seven of the nine ANG II values were previously published [24].

3.3. ANG II, ANG III, ANG IV and ANG V concentrations in normal canine heart

Table 1 shows raw data and corresponding mean ± SD of ANG II, ANG III, ANG IV and ANG V concentrations in nine left ventricles $n = 9$. Seven of the nine ANG II values were previously reported [24], these were included to compare with ANG III, ANG IV, ANG V and ir-ANG II determinations in this study. Measurements were made by the combined procedures of acidified ethanol tissue extraction, SPE, HPLC and RIA. Recovery of ANG II from the overall procedure as assessed by standard addition of ANG II was 42% ($n = 5$). The intra-assay precision of this method assessed by repeated measurements from a homogenous left ventricle pool was less than 15% ($n = 5$).

3.4. Immunoreactive ANG II (ir-ANG II) in normal canine heart

Ir-ANG II was measured from nine left ventricles having values of 57.2, 46.4, 45.3, 39.5, 28.3, 54.2, 49.9, 72.9 and 40.8 with a mean ± SD of 48.3 ± 11.9 fmol/g. Ir-ANG II was determined by hot acetic acid tissue extraction followed by RIA, without chromatographic processing. Recovery of this procedure, assessed by standard addition of ANG II, was 56% ($n = 5$). The intra-assay precision of ir-ANG II measurement was 6% ($n = 5$).

4. Discussion

Although ANG II antibody can differentiate between immunoreactive ANG II (ir-ANG II) from ANG I or ANG-(1–7) [20,21], it can not discriminate between the ANG II cascade members, peptides sequentially derived from ANG II. ANG II antibody cross-reacts not only with ANG II, but also with ANG III, ANG IV and ANG V. This ANG II antibody non-specificity is prevalent, regardless of the type or quality of the antibody produced. Both polyclonal [22] and monoclonal [23] ANG II antibodies were non-specific to ANG II. ANG II measurement by RIA, without chromatographic separation, measures ANG II and its immunoreactive peptides within its family. The ANG II antibody specificity, however, can be enhanced by the separation of immunoreactive members from each other prior to RIA.

HPLC was effective and efficient in separating immunoreactive ANG II members. Ion-pair reverse-phase HPLC was capable of separating ANG III, ANG V, ANG II and ANG IV with baseline resolution ($R \geq 1.5$) and capacity factor (k') range of $1.1 \geq k' \leq 2.8$. TEAF, as an ion-pair reagent, reduced retention time and improved symmetry of angiotensin HPLC peaks [24]. These angiotensin peaks were monitored with an UV-detector at a wavelength of 232 nm. Although the maximum absorbance of peptide bonds occurs at 187 nm [25], this wavelength was not practical in HPLC methods development of the angiotensins. Mobile phase additives, such as ion-pair reagents and organic modifiers, have an absorbance at this low wavelength as well. Nonetheless, it was desirable to use a wavelength as close as possible to the maximum adsorbance of peptide bonds to monitor ANG II and its fragments. The use of the UV-detector course absorbance adjustment allowed for the mobile phase absorbance to be zeroed at the working wavelength. Lower wavelength settings however, can be achieved with detectors having a wider span of course absorbance adjustment than the one that was used in this study. Desiderio and Cunningham [26] were capable of using a shorter wavelength with a different model UV-detector, in the separation of oligopeptides. In this study, the working wavelength of 232 nm provided a non-absorbing baseline and the detector was operated at lowest range (attenuation) setting of 0.001 AUFS. This was a minor disadvantage when compared to the resolution quality of ANG II cascade members within a relatively short elution time of 5 min. Such resolution and capacity factors of angiotensin peaks were a consequence of the TEAF ion-pair reagent used. Other laboratories have employed different mobile phase additives, such as, ammonium acetate [27,28], ammonium formate [29], ammonium phosphate [19], ammonium trifluoroacetate [28], phosphoric acid [30,31], triethylammonium phos-

phate [28,32,33], heptafluorobutyric acid [34,35], trifluoroacetic acid [36], trifluoroacetic acid and NaCl [37], sodium acetate [38] in the separation of immunoreactive angiotensins. However, the resolution and elution time obtained with TEAF was superior as compared to these ion-pair reagents in the separation of ANG II cascade members. An additional advantage of TEAF was its volatility, which allowed for easy drying of collected ANG II cascade fractions prior to RIA quantification. Collection windows for whole peaks were based on ANG II cascade retention times. Consistent retention times were observed between days, the coefficient of variation of ANG II, ANG III, ANG IV and ANG V retention times was less than 0.5%.

Collected ANG II cascade fractions from HPLC separation were quantified by RIA. Quantification of ANG II, ANG III, ANG IV and ANG V fractions were achieved by separate RIA standard curves (Fig. 2). These standard curves were generated with ANG II antibody and competitively challenged with ANG II tracer, ^{125}I-ANG II, to provide a measurable end point. The cross-reactivity of ANG II antibody facilitated the specific monitoring of HPLC separated immunoreactive ANG II peptide members. ANG II antibody was allowed to bind with ANG II cascade members separately. ANG IV standard curve was most sensitive while ANG II was the least, under similar experimental conditions. ED_{50} values for ANG IV, ANG III, ANG V and ANG II were 4.8, 6.6, 8.2 and 12.0 fmol/tube, respectively. This relative pattern of RIA standard curves was observed by another laboratory [36], however a reverse pattern was also observed [39]. This difference in relative sensitivity of standard curves may be attributed to different methods of producing ANG II antibodies, resulting in different binding affinities within the ANG II peptide family. Determined angiotensin values were within their standard curve ranges.

Prior to chromatographic processing or RIA, a suitable extraction procedure was required to release angiotensins from tissue into solution. Two methods have been proposed to extract angiotensins from tissue followed by SPE/HPLC/RIA or RIA alone. Acidified ethanol was initially used to extract insulin [41] and subsequently modified to extract angiotensins [17] from tissue. This acidified ethanol procedure was used to extract ANG II cascade members from canine cardiac left ventricle followed by SPE application. SPE further extracted and preconcentrated angiotensins prior to HPLC separation. ANG II, ANG III, ANG IV and ANG V fractions were collected and subsequently quantified by RIA. Measured percent of cardiac left ventricle ANG II, ANG III, ANG IV and ANG V were 64.5, 15.2, 11.5 and 8.9% of total ANG (sum of ANG II, ANG III, ANG IV and ANG V concentrations). The acidified ethanol tissue extraction procedure was lengthy, requiring centrifugation at about 32 000 $\times$ g and a tedious pH adjustment of supernatant. Artefacts from SPE in the measurement of ANG II, other than HPLC-derived shadowing, were reported [17,42]. A second method of angiotensin extraction from tissue, using hot acetic acid, was proposed [19,38]. Hot acetic acid extraction has been used to extract other regulatory peptides from the lung followed by RIA, without SPE pre-treatment [18].

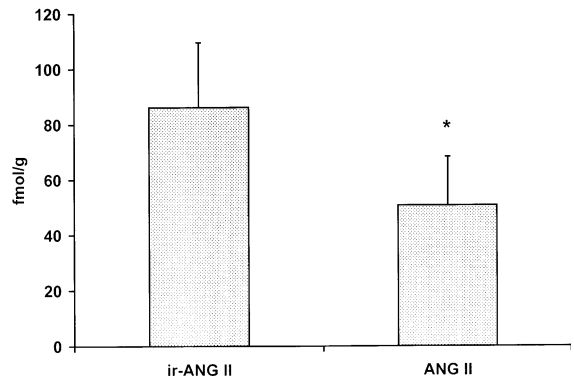

Fig. 3. Recovery rate corrected ir-ANG II and ANG II. ANG II was determined by acidified ethanol tissue extraction/SPE/HPLC/RIA, while ir-ANG II was determined by hot acetic tissue extraction/RIA. Data expressed as mean $\pm$ SD ($n = 9$), in fmol/g $^*P = 0.003$ versus ir-ANG II.

Shown in Fig. 3 are ir-ANG II and ANG II concentrations corrected for recovery rates, derived from hot acetic acid tissue extraction/RIA and acidified ethanol tissue extraction/SPE/HPLC/RIA, respectively. There was a significant

difference ($P = 0.003$) between left ventricular ir-ANG II and 'true' ANG II, corrected for recovery rates, of 86.2 ± 22.5 and 53.5 ± 16.2 fmol/g, respectively. ANG II, when separated from other immunoreactive peptide members prior to RIA, is considered to be 'true' ANG II [39]. On the other hand, ir-ANG II is a simultaneous measurement of all immunoreactive angiotensins to ANG II antibody quantified by an ANG II standard curve. It is conceivable that other standard curves, such as ANG III, ANG IV and ANG V may be used in the immunoreactive quantitation of angiotensins. These expressions of ir-ANG would be highly inaccurate since their concentrations are at least four times less than ANG II in the myocardium. Consequently, the difference between tissue ir-ANG II and ANG II is a reflection of the specificity of the methodology employed.

5. Conclusions

The non-specificity of ANG II antibody allows for its application in the specific radioimmunoassay measurement of ANG II cascade members, subsequent to HPLC separation. Triethylammonium formate is an effective and superior ion-pair reagent in reverse-phase HPLC separation of immunoreactive ANG II cascade peptides. ANG II, ANG III, ANG IV and ANG V separated isocratically in less than 5 min with baseline resolution. Normal canine left ventricular ANG II proteolytic fragments, ANG III, ANG IV and ANG V concentrations measured by acidified ethanol tissue extraction, SPE, HPLC and RIA are 5.3 ± 2.2, 4.0 ± 1.0, 3.1 ± 1.0 fmol/g ($n = 9$), respectively. The difference between left ventricular ir-ANG II and 'true' ANG II is significant ($P = 0.003$), corrected for recovery rates of 86.2 ± 22.5 and 53.5 ± 16.2 fmol/g, respectively. The combination of HPLC with RIA ensures the specific measurement of ANG II and its fragments. Moreover, the standard curves are of different sensitivities as reflected in their ED50 values. Consequently, the implication of the diverse biological effects of ANG II cascade members may require their characterization in diseases which involves their activation.

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