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Simplified method for quantitation of angiotensin peptides in tissue

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ABSTRACT

A simple method for extraction, separation, identification and quantitation of angiotensin-like immunoactivity from tissue is described. Homogenized acetic acid extracts of tissue samples were lyophilized and reconstituted in mobile phase. Separation was performed by reversed-phase high-performance liquid chromatography on a phenyl silica gel column with an eluent consisting of 20% acetonitrile in 0.1 *M* aqueous ammonium phosphate buffer, pH 4.9. Elution of standard peptides under isocratic conditions revealed clear resolution of angiotensin I, II and III and the (1–7) and (3–8) peptides. Recoveries of labeled angiotensin peptide standards from the extraction step were >90%. Radioimmunoassay of relevant peaks revealed detectable levels of angiotensin I-, II- and III-like immunoactivity in single rat hypothalami and brain stems.

INTRODUCTION

The renin–angiotensin system plays an important role in the maintenance of blood pressure and volume homeostasis in normotensive subjects without obvious cardiovascular disease and in various disease states, including systemic hypertension and congestive heart failure [1]. Renin (EN 3.4.23.15), a highly selective serine protease, cleaves its substrate, angiotensinogen, to produce the biologically inactive decapeptide, angiotensin I. The peptidyl dipeptide hydrolase angiotensin-converting enzyme (ACE; EN 3.4.15.1) removes the carboxyl terminal His–Leu from angiotensin I to produce the octapeptide angiotensin II. Angiotensin I and II are both rapidly hydrolyzed to inactive products in tissues by a number of proteolytic enzymes, the angiotensinases [2–4]. Angiotensin II is a potent pressor agent, acting directly via specific G-protein-coupled receptors in resistance vessels [5] and indirectly by stimulating the synthesis and release of the mineralocorticoid aldosterone from the adrenal glomerulosa [6] and the release of noradrenaline from sympathetic nerve terminals [7]. Angiotensin II has recently been shown to have a mitogenic and/or hypertrophic effect on vascular smooth muscle cells and cardiac myocytes [8,9], and these effects may contribute directly to the cardiovascular consequences of hypertension.

The classical view that the angiotensins function as circulating hormones has been challenged by recent findings of a complete renin–angiotensin system in a variety of tissues [10–12]. Further, it has been suggested that ACE inhibitors lower blood pressure by inhibiting the generation of angiotensin II in tissues, rather than in circulating plasma [13]. To define the role of the tissue renin–angiotensin system in hypertension and other disease states and to assess the importance of tissue

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ACE inhibition in the antihypertensive action of the ACE inhibitors, accurate and reproducible methods of quantifying the angiotensins in tissue are needed. The methods in current use for this purpose suffer from a number of problems that limit their application. Bioassay methods are generally too insensitive and non-specific for accurate quantitation of tissue angiotensin stores. Radioimmunoassay (RIA) procedures carried out on crude tissue extracts have produced inaccurate results because of interference and/or cross-reactivity with biologically inactive metabolites of the angiotensins, which are present in large quantities in tissues. An additional problem has been the instability of angiotensin during extraction from tissue, in large part due to cleavage of peptide bonds by tissue peptidases.

These limitations can successfully be overcome by use of gradient high-performance liquid chromatography (HPLC) in combination with RIA [14,15] after prior sample pretreatment with the solid-phase extraction (SPE) technique on disposable octadecylsilica gel (C_{18}) cartridges [16,17]. However, variable recoveries from the C_{18} cartridges [17], presumably due to inaccurate regulation of flow-rates [18], and the difficulties inherent in gradient elution techniques have limited the reproducibility and applicability of these methods.

The present study describes a simple two-step HPLC–RIA procedure for quantitating angiotensin I-, II- and III-like immunoactivity in tissue samples. Tissue was treated with boiling diluted acetic acid, and lyophilized extracts were applied to reversed-phase HPLC. Samples were eluted under isocratic conditions with a mobile phase consisting of acetonitrile in ammonium phosphate solution. Eluates were subjected to RIA for angiotensin I and II, which enabled the detection of angiotensin III-like immunoactivity due to the complete cross-reactivity of the antibody employed. This simple, sensitive and reproducible method is applicable for the measurement of tissue concentrations of angiotensin-like immunoactivity, which is required for studies of the renin–angiotensin system.

EXPERIMENTAL

Materials

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, a μBondapak Phenyl analytical column (300 mm × 3.9 mm I.D., 10 μm particle size), a Model 404 tunable UV detector (all from Waters Assoc., Milford, MA, USA) set at 200 nm, a one-channel recorder (Kipp and Zonen, BD 40, Alltech Assoc., Deerfield, IL, USA) and a Model 2110 fraction collector (Bio-Rad Labs., Richmond, CA, USA).

The ^{125}I-(Ile^5) angiotensin I and II (specific activity 2000 Ci/mmol) used for RIA and recovery studies were purchased from Amersham (Arlington Heights, IL, USA). Synthetic standard peptides, including angiotensin I, II and III and the (3–8) peptide, were purchased from Bachem Fine Chemicals (Torrance, CA, USA). Angiotensin (1–7) was kindly provided by Dr. Mahesh Khosla of the Cleveland Clinic Foundation (Cleveland, OH, USA). Ammonium phosphate was obtained from J.T. Baker (Phillipsburg, NJ, USA); other chemicals were purchased from Sigma (St. Louis, MO, USA). All chemicals used were of analytical grade.

Male Sprague–Dawley rats were obtained from Charles River Breeding Labs., (Wilmington, MA, USA).

Sample preparation

Male Sprague–Dawley rats (200–300 g body weight) were killed by decapitation without prior anesthesia. Brains were removed immediately and whole hypothalami and brain stems were dissected out on an ice-cold plate. Individual hypothalami and brain stems were frozen in liquid nitrogen and stored at −80°C until assay. Tissue samples (individual hypothalami and brain stems) were boiled in 1 *M* acetic acid (1–5 ml) for 15 min and then homogenized in a Waring blender for 30 s. The homogenates were centrifuged (30 000 *g* at 4°C for 30 min) and the supernatants were collected and lyophilized overnight to dryness, then reconstituted in 500 μl of the mobile

phase of the HPLC system. A 450-μl aliquot of each sample was injected into the HPLC system for separation of the angiotensin peptides.

HPLC separation

All separations were carried out at ambient temperature at a flow-rate of 1.0 ml/min. Mobile phases containing 10, 20, 30 and 40% (v/v) acetonitrile in 0.1 *M* ammonium phosphate and differing in pH were tested. The mobile phase consisting of 20% acetonitrile in 0.1 *M* ammonium phosphate buffer (v/v), pH 4.9, yielded optimal separations and was used in the remainder of the study. Eluate fractions (400 μl) were collected in plastic tubes (polypropylene, Sarstedt, Newton, NC, USA) in order to minimize sample absorption to the vial surface generally observed by use of glass-ware. Fractions with retention times corresponding to those of the angiotensin I, II and III standards were subjected to RIA immediately following elution from the column.

Radioimmunoassay

Antibodies to angiotensin I and II were raised in our laboratory in white New Zealand rabbits immunized against the peptides conjugated to poly-L-lysine, as previously described [19,20]. The cross-reactivity of anti-angiotensin I antiserum with angiotensin II and of anti-angiotensin II antiserum with angiotensin I was <0.5%; the cross-reactivity of anti-angiotensin II antiserum with angiotensin III was 100%. The sensitivity of the RIA for angiotensin I was 5 pg/ml and for angiotensin II, 2 pg/ml. Aliquots (100 μl) of each relevant fraction of column effluent were subjected to RIA immediately upon collection. Samples were assayed in duplicate without evaporation or other processing of the mobile phase. RIA for angiotensin I was carried out in polystyrene tubes (Sarstedt) and for angiotensin II, in polypropylene tubes (Sarstedt).

Recovery

The recovery of endogenous angiotensin II from the extraction step was estimated by two independent methods. In the first, [^{125}I]angiotensin II (6–9 · 10^5 cpm) was added to the tissue samples prior to boiling and ^{125}I-labeled material was measured in the HPLC fractions. In the second, 0.5 mmol of unlabeled angiotensin II dissolved in 200 μl of 1 m*M* HCl was added to the tissue samples prior to boiling, and angiotensin II was measured by RIA in the HPLC fractions. Values for angiotensin II in untreated brain tissue were subtracted from the values obtained for brain tissue with exogenous angiotensin II added in order to assess recovery.

The efficiency of the acetic acid boiling step in removing contaminating proteins from the samples was tested by measuring protein concentrations in tissue samples before and after the acid boiling step using the method of Lowry *et al.* [21].

RESULTS

Recovery

The recovery of [^{125}I]angiotensin II added to tissue samples was 93.1% (C.V. = 1.1% n = 6); the recovery of synthetic angiotensin II added to tissue samples was 90.4 (C.V. = 1.1%, n = 6). The [^{125}I]angiotensin II was eluted from the HPLC column as a single peak, suggesting that it was not degraded during sample handling. More than 90% of the protein in the sample was denatured in the acid boiling step and eliminated after centrifugation. Because the recovery of angiotensin II was almost complete, we did not correct our values for recovery.

HPLC separation

The relationship of H/V *versus* V (H = theoretical plate height of angiotensin II; V = mobile phase velocity, ml/min) was plotted to illustrate the influence of mobile phase velocity on analysis time (Fig. 1). The influence of different acetonitrile concentrations and pH values on the capacity factor k' of the synthetic angiotensin peptides is depicted in Fig. 2 and Table I. As a consequence of these investigations, a mobile phase consisting of 20% acetonitrile in 0.1 *M* ammonium phosphate buffer, pH 4.9, at a flow-rate of 1.0 ml/min proved to be optimal for separation of the synthetic angiotensin peptides. Changing the pH of the buffer in the mobile phase did not in-

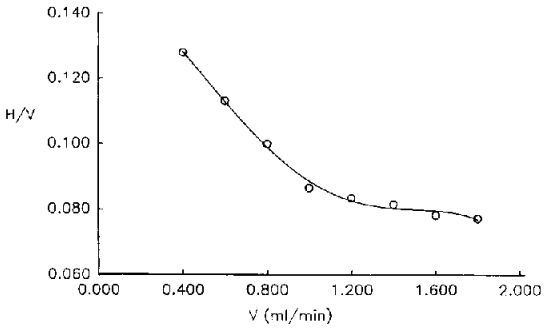

Fig. 1. Plots of H/V *versus* V illustrating the influence of mobile phase velocity on analysis time. H = theoretical plate height of angiotensin II; V = mobile phase velocity in ml/min.

terfere with the immunoreactivity or alter the retention order of the angiotensin peptides.

Under the isocratic conditions described above, clear separation of unlabeled standards of angiotensin I, II, III, (1–7) and (3–8) peptides was achieved (Fig. 3). Approximately 5–10 ng of each peptide were applied to the column.

Fig. 4 shows a typical elution pattern of the extract of an individual rat hypothalamus. Three distinct peaks of immunoreactive material eluted with the same retention times as synthetic angiotensin I, II, and III. An additional unidentified peak of immunoreactivity with anti-angiotensin II antibody was present in fractions 6–8. Table II shows mean (± S.E.M.) values for angiotensin I-, II- and III-like immunoactivity in hypothalami and brain stems derived from six Sprague–Dawley rats.

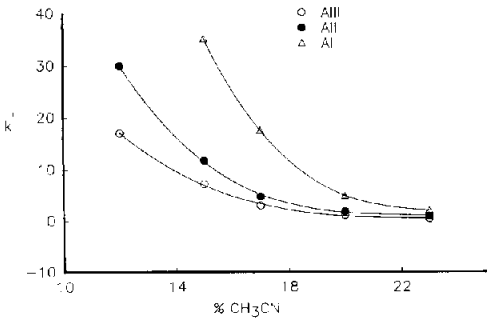

Fig. 2. Effect of acetonitrile content (v/v) in the mobile phase on capacity factor k'. Column: Waters μBondapak Phenyl (300 mm × 3.9 mm I.D., 10 μm particle size). Flow-rate: 1.0 ml/min. Temperature: 25°C.

TABLE I

RETENTION TIMES OF ANGIOTENSIN PEPTIDE STANDARDS ON A μBONDAPAK PHENYL COLUMN WITH MOBILE PHASES OF DIFFERENT pH VALUES

For chromatographic conditions, see text. AI = angiotensin I; AII = angiotensin II; AIII = angiotensin III; A (1–7) and A (3–8) = peptides containing the (1–7) and (3–8) sequences of angiotensin II, respectively.

pH	Retention time (min)				
	AI	AII	AIII	A (1–7)	A (3–8)
3.3	17.6	10.2	6.8	4.1	12.4
4.9	14.6	7.2	5.4	4.0	8.6
6.5	23.8	7.4	6.8	4.0	10.2

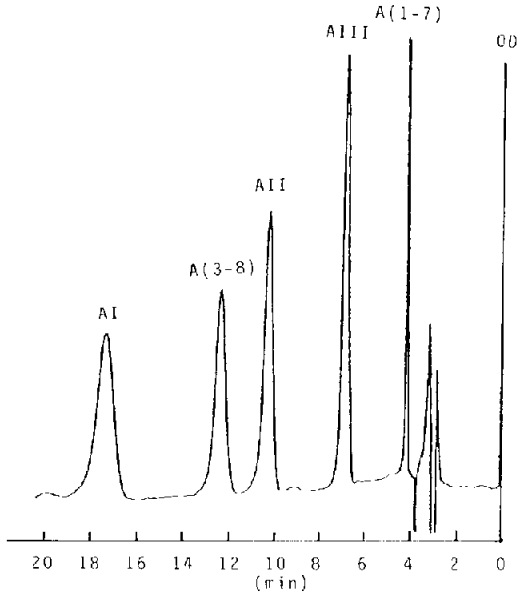

Fig. 3. HPLC separation of synthetic angiotensin peptides on a μBondapak Phenyl column (300 mm × 3.9 mm I.D., 10 μm particle size) under the isocratic conditions described in the text (pH 4.9). UV detection at 200 nm. For other chromatographic conditions, see text.

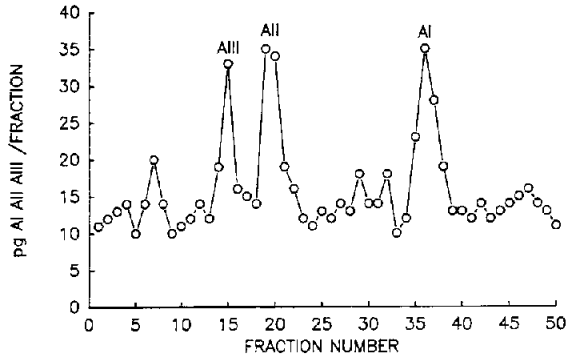

Fig. 4. HPLC separation and RIA measurement of angiotensin-like immunoactivity from hypothalamus. Angiotensin-like immunoactive peptides extracted from rat brain were separated by HPLC and identified by RIA. Fraction volumes were 400 μl. pg AI, AII, AIII = picograms of angiotensin I-, angiotensin II- and angiotensin III-like immunoactivity.

TABLE II

ANGIOTENSIN-LIKE IMMUNOACTIVITY IN BRAIN REGIONS OF SPRAGUE–DAWLEY RATS

Values are expressed as means; values in parentheses are C.V.s (%). AI = angiotensin I-like immunoactivity; AII = angiotensin II-like immunoactivity; AIII = angiotensin III-like immunoactivity.

Brain area	*n*	Immunoreactivity (pg/g wet tissue)		
		AI	AII	AIII
Hypothalamus	6	1847 (18.6)	691 (29.1)	430 (48.4)
Brain stem	6	202 (32.7)	68 (23.4)	57 (34.4)

DISCUSSION

The present study describes a simple HPLC–RIA method for resolution and subsequent quantitation of angiotensin I-, II- and III-like immunoactivity in biological materials that requires neither a tedious prepurification procedure of the peptides nor gradient elution conditions of the peptides from the HPLC column. Most published procedures for determination of the angiotensin peptides in biological fluids or tissue employ C_{18} cartridges for preliminary purification of the peptides prior to separation and assay [16,17]. The solid-phase extraction of peptides from acidified tissue homogenates on C_{18} cartridges has been reported to have a high recovery rate for peptides of interest, to preserve the biological properties of these peptides and to protect the peptides from degradation by proteases. However, experience in our own laboratory and others [17] has indicated that recoveries of angiotensin from tissue samples extracted on C_{18} cartridges are highly variable (but correctable). Dried residues of eluates from C_{18} cartridges have been found to decrease the binding of [^{125}I]angiotensin II to anti-angiotensin II antibody in assay tubes, causing high blank values. Thus, materials eluted from the cartridges either interfere with binding or destroy the tracer. Blank values have been shown to vary substantially among lots of C_{18} cartridges, thus contributing to high inter-assay C.V.s [17]. Large inter-assay variation is a particular problem at low concentrations of peptide, as is the case with angiotensin II in plasma and brain tissue.

Affinity chromatography utilizing purified anti-angiotensin II antiserum coupled to a solid support has been used as an alternative method of isolating and concentrating angiotensin II-like immunoactive material from biological samples [22,23]. Although this method is useful for concentrating angiotensin II and III from extracts of tissue or culture media, it does not concentrate other angiotensin peptides, such as angiotensin I or the (1–7) peptide, which do not bind well to most anti-angiotensin II antisera. Affinity chromatographic methods have the additional disadvantages of extensive consumption of costly antisera and solid support resins, limited sample throughput, a requirement for substantial biochemical expertise for the synthesis and regeneration of affinity columns and the characterization of antisera, and variable results, depending on the characteristics of available antisera. Further, the question arises if antibody coupling to the solid-phase resin completely preserves its original

immunogenic properties. Cross-linking of the antibody during its tagging to the solid-phase support may contribute to a significant change in the original epitope and thus either altered antigen-binding or cross-reaction characteristics must be taken into consideration. For these reasons, affinity chromatographic methods have limited feasibility in measuring low concentrations of angiotensin peptides in studies of the regulation of the tissue renin–angiotensin system. Alternative methods include the use of a protein A Sepharose conjugate or the immunobeads second antibody technique. However, these methods suffer from the high costs of the required reagents.

To our knowledge this is the first study to describe an HPLC system in which clear separation of the biologically relevant angiotensin peptides derived from tissue stores is achieved by application of isocratic elution conditions. Elimination of a requirement for gradient elution conditions greatly simplifies the procedure and enhances its reproducibility, because differential evaporation of volatile solvents in gradient elution systems can substantially alter separation conditions and introduce errors into the calculations of final results. In our procedure, fractions of effluent from the HPLC column are subjected to RIA immediately upon collection, thus minimizing evaporation of the volatile part of the column effluent, acetonitrile, and consequent variability in concentrations of angiotensin peptides among fractions of effluent. The reversed-phase μBondapak Phenyl column provides clear resolution of the angiotensin peptides, which differ in chain length by only one or two amino acid residues. Peptides are separated based on differences in their degrees of ionization, which depend, in turn, on the pH of the mobile phase and on pH-independent mechanisms such as the polarity and character of their hydrophobic side-chains and the eluotropic strength of the organic solvent of the mobile phase [14,24]. In preliminary experiments, we tested the effects of altering the eluotropic strength (acetonitrile content) and pH of the buffer in the mobile phase on the elution profile of standard angiotensin peptides. A mobile phase consisting of 20% acetonitrile in 0.1 *M* ammonium phosphate buffer (v/v), pH 4.9, yielded clear separation of all of the model peptides tested. Retention times were low: all peptides were eluted from the column in less than 20 min. All immunoreactive material derived from peptide standards and endogenous brain angiotensin eluted with the same retention times as synthetic angiotensin I, II and III, and 90% of standard angiotensin II added to tissue samples was recovered in eluates from the HPLC column. The high resolution and excellent recovery characteristics of this system contrast with earlier reports of poor resolution of angiotensin peptides and failure of immunoreactive material to co-elute from reversed-phase HPLC columns with standard peptides [14,15,25]. Elimination of the preliminary purification step on C_{18} cartridges and use of a Phenyl stationary phase for the HPLC column, with its reduced hydrophobicity compared to C_{18} columns and enhanced selectivity and resolution for basic peptides at low pH, may account for the improved resolution and high reproducibility of our method. The concentrations of angiotensin II- and III-like immunoreactivity in brain tissue observed in the current study are in the same range as previously reported [14,15]; the concentration of angiotensin I-like immunoreactivity is somewhat higher. Future studies will apply this simple, novel and highly reproducible analytical method to examine profiles of angiotensin-like immunoreactivity in tissues derived from normotensive subjects without obvious cardiovascular disease, from subjects with a variety of disease states and from subjects treated with various therapeutic agents, including ACE inhibitors and angiotensin II receptor antagonists.

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