Extraction and Measurement of Circulating Angiotensins I and II

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We describe a method for simultaneous extraction of angiotensins I and II from human blood. Blood was diluted with 10 volumes of isotonic saline containing ammonium ethylenediaminetetraacetate, and the angiotensin peptides were extracted by cation-exchange chromatography. The dilution effectively inhibits in vitro production of angiotensin I, conversion of angiotensin I to angiotensin II, and degradation of the peptides. Analytical recovery of exogenous angiotensin I or II added to whole blood in physiological concentrations was essentially complete. The extraction procedure removes blood and plasma components that interfere with radioimmunoassay of angiotensins, thus eliminating the need for correction for nonspecific interference. Circulating angiotensins I and II, measured in patients with essential hypertension before and after a week of diuretic administration, increased in parallel with plasma renin activity.

Additional Keyphrases: radioimmunoassay · essential hypertension · renin and angiotensinase activity · heart disease

Reportedly, there are changes in the metabolic clearance rate and possibly the mechanism of degradation of the angiotensin peptides in hypertensive disease (1), in situations of altered sodium balance (2), and after diuretic administration (3). The end product of the renin/renin-substrate reaction, angiotensin I, and the effector agent of the renin/angiotensin system, angiotensin II, must both be measured in blood before the overall in vivo activity of the circulating renin system can be evaluated. Measurement of circulating angiotensin peptides requires attention to several important methodological details. In the case of both angiotensins I and II, angiotensin-degrading enzymes and converting enzyme(s) in blood or plasma must be completely inhibited, and renin completely and instantaneously inhibited.

Current methods for measuring angiotensin I and for separately measuring angiotensin II in human blood or plasma achieve the above objectives to various degrees.

Our purpose was to develop a method for simultaneously extracting angiotensins I and II from blood that would meet all of these important requisites.

Materials and Methods

Sample Collection and Preliminary Treatment

Human peripheral venous blood (25-ml specimens) was collected into a plastic syringe from ambulatory subjects on an unrestricted diet. To inhibit the enzymic activity of renin, converting enzyme, and angiotensinases, we tried treating 20-ml aliquots of the blood by immediately dispersing it into either 100 ml of 95% ethanol or 100 or 200 ml of 0.15 mol/liter saline with ammonium ethylenediaminetetraacetate (EDTA) added (96/4 by vol), pH 7.4 (4). In either case, the diluent was contained in a 250-ml plastic centrifuge bottle, cooled in an ice bath.

Procedures

Extraction of angiotensin. The 250-ml centrifuge bottle containing the dispersed blood sample was centrifuged (2 °C, 15 min, 5000 × g). To determine whether significant binding of angiotensin occurred, a measured aliquot of the supernate was removed or the precipitate washed with an additional 30 to 50 ml of alcohol or saline. The saline extracts were applied directly to columns containing cation-exchange resin (see below). The alcohol extracts were taken to dryness (below 40 °C) with a rotary evaporator, dissolved in 0.15 mol/liter saline (10–20 ml), and applied to the ion-exchange columns. Plastic syringes (30 ml) with plastic stopcocks were used as columns and contained 10 to 15 ml of Dowex resin (AGW X2, 100–200 mesh; BioRad Laboratories) precycled by the method of Boucher et al. (4). After application of the blood extracts, the columns were washed successively with 25 ml of ammonium acetate (0.2 mol/liter, pH 6.0) and 20 ml of distilled water (4). The columns were then washed with 25 to 30 ml of acetic acid/water (10/90 by vol) (5) or 25 ml of 0.1 mol/liter HCl. The angiotensin peptides were subsequently eluted with 25 ml of NH₄OH in concentrations ranging from 0.4 to 1.0 mol/liter, which was collected into a
100-ml siliconized round-bottom flask. The pH of the eluted peptides was adjusted to between 6.0 and 6.5 with glacial acetic acid. In some control experiments, samples were heated for 10–15 min in a boiling water bath. All samples were then lyophilized.

**Plasma renin activity.** The techniques for plasma renin activity determination have been published (6).

**Radioimmunoassay.** Antisera to angiotensins I and II were produced in New Zealand white rabbits as previously described (7, 3) and used at a final dilution of 1/30 000 and 1/100 000, respectively. Monoiodinated angiotensin I and II (Asp'-Ileu5) were obtained from Schwarz/Mann and used without further purification because the undiluted antibodies could bind in excess of 95% of the labeled peptide (5000 cpm/ml). We have previously published our methods for the angiotensin radioimmunoassays (8). Standard angiotensins I and II were obtained from the National Institute for Biological Standards and Control, Hampstead, England. High- and low-concentration control samples of synthetic angiotensins I or II were included in each assay.

Synthetic angiotensin II (Asp'-Ileu5) or the synthetic tetradecapeptide renin substrate (Schwarz/Mann) did not cross react with the angiotensin I antiserum. The angiotensin II antiserum showed no cross reactivity with angiotensin I, but reacted equally well with several unpurified lower molecular weight homologs of angiotensin II obtained from Schwarz/Mann, including des-Asp1-angiotensin II (heptapeptide), des-Asp1-Arg2 (hexapeptide), and des-Asp1-Arg2-Val3 (pentapeptide).

For radioimmunoassay of unknown samples, the lyophilized Dowex column eluate was taken up in 3.0 ml of tris(hydroxymethyl)aminomethane acetate buffer (0.1 mol/liter, pH 7.4) containing 1 g of lysozyme (Sigma) per liter. Each unknown was assayed in triplicate at two dose levels (0.1 and 0.2 ml).

**Purification of synthetic angiotensin I and II.** Angiotensin I and II (Asp1-Ileu5, Schwarz/Mann) were partly purified by column chromatography on the Dowex resin (4) and electrophoresis on polyacrylamide gel (pH 8.4) (9). The gels were extruded with a Gilson Alichogel. Pressor activity (10) and immunological activity of the partly purified material were compared to those of the standard peptides.

**Results**

**Purification of Synthetic Peptides**

The relative activity of synthetic angiotensin II as compared to the standard angiotensin II, determined by bioassay and radioimmunoassay, was not affected by the Dowex column chromatography. In contrast, we found that although the total pressor activity of angiotensin I remained constant, the total immunological activity of the sample was less after chromatography. The quantity of immunologically active material removed by the cation-exchange resin appeared to differ with various lots of the synthetic peptide (10–40%).

Recycling the angiotensins I or II over a second Dowex column did not change the bioassay/radioimmunoassay ratio. After electrophoresis on polyacrylamide gel, one band of immunologically active material was found for each peptide. These purified peptides could not be distinguished from the standard peptides by bio- or radioimmunoassay.

**Comparison of Ethanol and Saline/EDTA for Angiotensin Extraction**

The measured amounts of angiotensin I or II extracted from pooled samples of peripheral blood collected in ethanol varied; replicate determinations showed as much as a five- to sixfold difference, a lack of reproducibility not influenced by the number of times the blood precipitate had been washed. After pooled blood was dispersed into saline/EDTA, however, replicate determinations of angiotensins I and II were reproducible, averaging 33.6 ± 2.4 (SEM) ng/liter and 14 ± 0.9 ng/liter (n = 6), respectively. Washing the erythrocytes with saline/EDTA (30 to 50 ml) did not change the quantity of extractable angiotensins I or II. In a separate blood pool, the concentration of angiotensin II extractable by saline/EDTA (nonwashed cells) and in plasma was compared. After the plasma was separated from pooled blood, the angiotensin II values averaged 23.9 ± 1.6 ng/liter vs. 22.2 ± 3.1 ng/liter in the saline extract.

**Separation of Angiotensin I and II from Components that Interfere with Radioimmunoassay**

Addition of plasma to the radioimmunoassay standards caused a dose-dependent decline in the percent bound, which did not parallel the standard curve generated with standard angiotensins I or II in buffer alone. This nonparallel displacement of the standard curve was also observed with blood extracts eluted from Dowex columns, even though the columns had been washed with distilled water (4) or acetic acid/water (10/90) (5) before elution of angiotensin. Column eluates that had been boiled for 10 min and centrifuged (15 000 X g) before lyophilization still caused nonparallel displacement of the radioimmunoassay standard curve. Parallel displacement was observed, however, if the Dowex columns, after the saline/EDTA extract was applied, were washed with 25 ml of 0.1 mol/liter HCl and then 20 ml of distilled water before elution of angiotensin with 1.0 mol/liter NH4OH. The NH4OH fraction was then heated in a boiling water bath for 10 min.

The nonparallel displacement observed with column eluates obtained before the hydrochloric acid wash was shown to be caused by soluble components in the eluate, which somehow prevented binding of 125I-labeled angiotensin to the charcoal. The acetic acid, hydrochloric acid, and the final ammonium hydroxide fractions (obtained after hydrochloric acid washing of the Dowex column) were lyophilized and dissolved in the pH 7.4 buffer. These three fractions were then incubated with labeled angiotensin I or II for 48 h and free angiotensin
plasma stored on ice, blood samples were divided and either centrifuged to obtain plasma or dispersed into saline/EDTA. Angiotensin I in plasma was significantly greater (82.3 ± 4.3 ng/liter) than observed in the saline extract (68.3 ± 5.2 ng/liter), even though both the plasma and the saline extracts were column chromatographed within 30 min.

Recovery of Exogenous Angiotensin I and II from Whole Blood

Chromatographically purified synthetic angiotensin (50 and 100 ng of angiotensin I per liter and 25 and 50 ng angiotensin II per liter) were added to blood. The percentage recovery of angiotensin I was 100 ± 10.8% (SD) at 50 ng/liter and 96 ± 7.4% at 100 ng/liter. Recovery of angiotensin II was 95 ± 15% at 25 ng/liter and 94 ± 16% at 50 ng/liter (n = 6).

Circulating Angiotensin in Essential Hypertensive Subjects

Circulating angiotensin I and II were determined in 14 men with benign essential hypertension. For control studies, all of them were taken off medication for a month. They were then given an oral diuretic (metolazone, 5 mg, or hydrochlorothiazide, 100 mg daily) for a week. The results of this study are shown in Table 1. Plasma renin activity measurements are listed for comparison. All three analytes significantly increased (P < 0.005) in response to diuretic administration.

Discussion

In the present study, ethanol extraction of endogenous angiotensin resulted in a large variability in the results. The reasons for this are not entirely apparent but may be related to nonspecific binding or entrapment of angiotensin to the alcohol-denatured erythrocytes or plasma proteins. Extensive washing of the precipitate did not significantly increase analytical recovery of added angiotensin. Although some investigators have collected blood directly into cold alcohol (11-13) thus preventing renin, angiotensinase, and converting enzyme activities, the reported analytical recoveries of angiotensin by this technique have been relatively low and variable. Osborne (12) reported only 30% recovery and Waite (13) reported recoveries that averaged 50% (range 40–60%). Catt et al. (11) reported somewhat higher values for recovery (65 to 75%) when
tracer quantities of radiolabeled angiotensin were used to correct for recovery. We also have found satisfactory and reproducible recovery of added angiotensin from rat blood on alcohol extraction (14) if we added high pharmacological concentrations of angiotensin.

Extraction of blood into 10 volumes of chilled saline/EDTA appears to serve the same function as collection in ethanol—namely, to prevent renin, converting enzyme, and angiotensinase activities, at least during the time required for blood processing. Effective inhibition of renin activity was confirmed by showing no change in angiotensin I for as long as 1 h after blood collection. Renin activity was significant at 60 min when blood was collected into only five volumes of saline/EDTA and also at 30 min when whole plasma was extracted. Continued plasma renin activity at low temperatures has previously been described by Malvano et al. (15) and Emanuel et al. (16). We have also observed no change in total angiotensin II concentrations in samples of pooled blood after dilution in 10 volumes of cold saline/EDTA, even though the samples were allowed to stand on ice for 90 min. Apparently, the EDTA and low temperature are sufficient to inhibit the angiotensin converting enzyme and angiotensinases, because the measured concentrations of angiotensin II in diluted blood and in plasma were not different, suggesting that there is little or no binding of angiotensin to erythrocytes, although simple dilution of blood may be sufficient to remove the bound peptide.

Several reports describe either characterization (18) or isolation (7) of proteins that interfere with the radioimmunoassay of angiotensin I or II. Interference with angiotensin radioimmunoassay by plasma proteins may be related to the particular antisera used for the assay (15). More often, however, interference has been reported to be a variable characteristic of the individual plasma sample being assayed (18, 19), and attempts to compensate for this interference have been made (18, 20, 21). Our antisera also cross react with plasma proteins. After extraction by our technique, however, the patient-dependent interference with radioimmunoassay is eliminated, because there is a parallel and proportional decrease in the percent bound on the standard curve when increasing volumes of blood extract are assayed. Thus there is no need to run either separate controls for each sample or add angiotensin-free plasma to the standard curve.

Cation-exchange column chromatography of the synthetic angiotensin I was a prerequisite for our recovery studies. This precycling through the extraction procedure apparently removed immunologically active but biologically inert material that did not behave similarly to angiotensin on the column. These substance(s) may be large C-terminal fragments of angiotensin I, which may cross react with angiotensin antisera as do such fragments of angiotensin II (22). Use of synthetic peptide as supplied by the manufacturers would have led to erroneous results for recovery, because 10 to 40% of the immunologically active material was not intact angiotensin I.

We have determined the total quantity of biologically active angiotensin peptides recovered from pooled blood samples as compared to the values of angiotensin I and II as determined by separate radioimmunoassay. Because rat pressor assay requires nanogram quantities of angiotensin for accurate quantitation, it was necessary to pool column eluates from many blood samples. This pool contained approximately 8 μg of angiotensin per liter, as measured by bioassay. By radioimmunoassay, we found 6.1 μg of angiotensin I and 1.3 μg of angiotensin II per liter. This result indicates that the sample contained predominately intact angiotensin I and angiotensin II and is compatible with the observations of Semple and Morton (7) suggesting that about 80% of the immunoreactive material in venous blood that cross-reacts with the angiotensin II antibody is the intact peptide. It may be, however, that our separation methods have largely eliminated immunologically active metabolic fragments of angiotensins I and II, although their presence cannot be excluded with assurance.

The concentrations of circulating angiotensin I that we found for untreated patients with essential hypertension are somewhat higher than those reported in normal subjects by Waite (13), who used alcohol extraction. The data after diuretics, however, are in remarkably close agreement in the two studies. Angiotensin I concentrations in plasma found by Emanuel et al. (16) and by Cohen et al. (21) were significantly higher than we observed, probably because of continued renin activity even in cold plasma. The concentrations of circulating angiotensin II in our group of patients agreed well with the values observed by Catt et al. (11) using alcohol. Because we saw no significant binding of exogenous angiotensin II to erythrocytes, we think that endogenous concentrations in blood can be validly expressed as plasma concentrations by correcting for hematocrit. The concentrations of angiotensin II in blood that we found are about twofold higher than the values for plasma reported by others (23–25), but are significantly lower than observed by Gocke et al. (20), who measured angiotensin II in nonextracted plasma of normal subjects. As expected, however, there was an acute increase in circulating angiotensin I and II after a week of therapy with diuretics, which paralleled the increase in plasma renin activity.

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References


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