Rapid, Simplified Radioimmunoassay of Arginine-Vasopressin and Atrial Natriuretic Peptide in Plasma

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We have improved a radioimmunoassay for arginine-vasopressin (AVP) and atrial natriuretic peptide (ANP) by using Sep-Pak C18 cartridges to extract AVP and ANP from acidified plasma. The analytes are co-eluted by use of a mobile phase consisting of 1,2-dimethoxyethane and 40 g/L aqueous trifluoroacetic acid (95/5, by vol). After rapid evaporation of the solvents, AVP and ANP are assayed by a nonequilibrium radioimmunoassay method in which commercially available antibodies and radiolabeled antigens are used. The bound fractions are separated from the free by use of polyethylene glycol with human gamma globulin and rabbit anti-human IgG as the second antibody. This results in very low nonspecific binding: 0.44% for the ANP assay, 0.70% for AVP. The minimum detectable amount of ANP is 0.39 pg per tube; for AVP, it is 0.13 pg per tube. Compared with other published methods, this method is substantially more reliable, economical, and easily established in a clinical chemistry laboratory.

Additional Keyphrases: heart disease · congestive heart failure

Congestive heart failure is characterized by impaired cardiac output and increased peripheral vascular resistance. Because vasodilator therapy with hydralazine and isosorbide dinitrate improves survival in these patients, perhaps increased peripheral vascular resistance contributes to poor prognosis in congestive heart failure.

Strong evidence suggests that patients with heart failure have increased circulating concentrations of arginine-vasopressin (AVP) (1, 2). Administration of a competitive inhibitor of AVP decreases peripheral resistance and improves cardiac output in these patients (3). Concentrations of atrial natriuretic peptide (ANP) in plasma are also increased in patients with congestive heart failure (4). ANP inhibits renin production (5), enhances the glomerular filtration rate (5), and improves natriuresis (6). Thus, ANP may have an important counterbalancing action on the detrimental vasoconstrictor effects of AVP in heart failure. We believe that concurrent measurement of AVP and ANP could be of value in evaluating the role of these peptides in the prognosis of patients with heart failure.

Assays of the peptides AVP and ANP are complicated by the extremely low concentrations of both in plasma, requiring the use of highly sensitive and specific antibodies, and extraction of plasma. Extraction of AVP and ANP from biological fluids is complex, with variable analytical recoveries (7–13).

Here we report a rapid, simple method for simultaneously extracting ANP and AVP from plasma with a Sep-Pak C18 cartridge. Extracted samples are rapidly lyophilized under reduced pressure in a centrifuge, and recoveries are consistently high. For the radioimmunoassay, we used commercially available antibodies, which, in our experience, displayed no stability problems (12).

Materials and Methods

Sample preparation: Blood was collected in tubes containing Na2EDTA (final concentration, 4.5 mmol/L), then centrifuged (4 °C, 15 min, 2000 × g). We then added 0.1 mol/L phenylmethylsulfonylfluoride (PMSF) solution to the plasma, to give a final concentration of 5 mmol/L, and stored the samples at −80 °C.

Sample extraction: After attaching 12 Sep-Pak C18 cartridges (Waters Associates, Milford, MA) to a vacuum manifold (Lida, Bensenville, IL), we washed each in sequence with 10 mL of tetrahydrofuran, 50 mL of methanol, and 20 mL of de-ionized distilled water. To extract plasma samples or samples with added ANP (Peninsula Labs, Belmont, CA) or added AVP (United States Pharmacopeia, Rockville, MD), or both, we used a modification of the method of La Rochelle et al. (14), as follows.

Thaw samples at room temperature and mix well. Acidify 2-mL aliquots of plasma to pH 5.5 by adding 100 μL of acetic acid (2.5 mol/L, pH 5.5), then centrifuge at 4 °C for 30 min at 40 000 × g. Pass 2 mL of this supernatant liquid through the Sep-Pak cartridge at the rate of 1 mL/min (using suction), wash the cartridge with 20 mL of water, and dry the column by suction. Elute AVP and ANP from the washed cartridges by adding 4 mL of a mixture of 1,2-dimethoxyethane and 40 g/L aqueous trifluoroacetic acid (95/5, by vol). Aliquot the eluate into polypropylene tubes (2 × 1.5 mL for AVP and 2 × 0.5 mL for ANP) and dry the aliquots in a centrifuge (we used one from Savant, Hicksville, NY) under reduced pressure. Reconstitute the samples in 25 μL of methanol, followed by 100 μL of assay buffer (see below).

To prepare assay buffer, incubate for 4 h at 56 °C, with mixing, 20 g of bovine serum albumin (BSA) in 50 mmol/L phosphate buffer (pH 7.4) containing 5 mmol of PMSF, 0.1 mol of NaCl, 10 mmol of EDTA, and 0.1 mL of Triton X-100 surfactant per liter. After incubation, dilute 10-fold (to 2 g of BSA per liter) with the same buffer. Then add neomycin sulfate (2 g/L) and sodium azide (1 g/L) and adjust the pH to 7.4. Filter the buffer through Whatman no. 1 filter paper (Whatman, Clifton, NJ) before use.

Radioimmunoassay (RIA): Both anti-AVP (Calbiochem, San Diego, CA) and anti-ANP (Peninsula Labs) antibodies were diluted to bind about a third of the total radiolabeled antigens added in the absence of unlabeled antigen (B0). The quantities of labeled antigens used were 1.6 pg per tube for 125I-labeled AVP and 6.0 pg per tube for 125I-labeled ANP (both from Amersham, Arlington Heights, IL, and each with specific activities of 2000 Ci/mol). The samples for the standard curve and the extracted antigens were assayed simultaneously, in triplicate.
Table 1. Mean Percentage and SD for Antigen Recoveries

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<th>Antigen added, * pg/mL</th>
<th>Mean recovered (and SD), %</th>
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<tr>
<td></td>
<td>AVP</td>
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\(^a\) Added to 1 mL of outdated plasma from the blood bank. Endogenous content of this stored plasma was 1.2 pg of AVP and 1.6 pg of ANP per milliliter.

\(^b\) Percentage of AVP and ANP recoveries determined by using \(^125^I\)-labeled AVP and ANP added to the plasma.

\(^c\) n = 8. All others, n = 16 determinations each.

To start the RIA, add 100 \(\mu\)L of diluted antisera to all tubes containing the standards and the samples (100 \(\mu\)L) except the total count and the nonspecific binding tubes. After 16 h of incubation at 4°C, add 100 \(\mu\)L of labeled antigen to all the tubes. After an additional 16 h of incubation at 4°C, add 50 \(\mu\)L of an equivolume mixture of goat anti-rabbit gamma globulin [2 arb. (supplier’s) units/mL] and human gamma globulin (40 g/L) and 1 mL of polyethylene glycol (PEG, \(M_w\) 8000; J. T. Baker Chemical Co., Phillipsburg, NJ) reagent, 25 g per liter of Tris buffer (10 mmol/L, pH 7.4). Dilute the goat anti-rabbit gamma globulin (Calbiochem) and human gamma globulin (Sigma) with the Tris buffer and store them at -20°C before use. Vortex-mix briefly, then centrifuge the samples at 4°C for 30 min at 2000 \(\times\) g. Decant the liquid from the tubes, aspirating any adhering to the sides with Pasteur pipettes, and count the radioactivity in the pellet. Calculate the logit of the response and the log of the concentrations of standards and construct the standard curve by an unweighted regression analysis.

We determined analytical recoveries by doing parallel extractions of 1, 2, 4, and 8 pg of AVP and 3.12, 12.5, 25, and 50.0 pg of ANP added to different 1-mL aliquots of plasma (Table 1).

Physiological studies: To test the changes in plasma immunoreactive AVP and ANP in response to known physiological stimuli, seven normal volunteers (four men, three women) underwent a 24-h dehydration test after an initial water loading (20 mL per kilogram body weight). At the end of each period, blood samples were collected for the determination of plasma osmolality (\(P_{\text{o}}\)), plasma AVP, and ANP. Subjects were supine for 15 min before venipuncture and maintained their normal work schedules. AVP concentrations were also measured in the plasma of two patients with a three-year history of neurogenic diabetes insipidus.

Statistical analysis: All physiological values are expressed as the mean ± 1 SEM. The unpaired Student’s t-test (two tailed) was used for all the comparisons. In all cases a \(P\) value < 0.05 was considered significant.

Results

Sample extraction: Recovery of \(^125^I\)-labeled AVP and of \(^125^I\)-labeled ANP (both about 40 000–60 000 cpm) added to 2 mL of plasma averaged 89.1 ± 11.4% (n = 8) and 84.3 ± 6.7% (n = 8), respectively (mean ± SD). About 2.9% of the AVP and 2.4% of the ANP applied to the column were not retained. Sep-Pak cartridges can be re-used two or three times by washing them sequentially with tetrahydrofuran, methanol, and water, a treatment that removes all the bound radioactive counts from the matrix. When physiological concentrations of AVP or ANP standard were added to plasma and carried through the extraction and RIA, there was a linear relationship between measured and added AVP and ANP concentrations (Table 1).

At pH 5.5, both ANP and AVP were bound to the C\(_{18}\) matrix, but at neutral pH only about 70% of the AVP was retained on the cartridge. When HCl was used for acidification of plasma, the lyophilized plasma extracts produced a large, dark, relatively insoluble residue as opposed to a clear, small, easily soluble pellet with acetic acid.

Assay performance: Final reagent concentrations of 18 g of PEG per liter, 1.5 g of human gamma globulin per liter, and 1.5 units of goat anti-rabbit gamma globulin produced low mean nonspecific bindings, 0.70% (SD 14.8%, n = 8) for AVP and 0.44% (SD 14%, n = 8) for ANP. Two milliliters of water extracted with 4 mL of the 1,2-dimethoxyethane and 40 g/L of aqueous trifluoroacetic acid mixture (95/5, by vol) produced blank values equivalent to 0.34 pg per tube for ANP and 0.12 pg per tube for AVP. Addition of 25 \(\mu\)L of methanol or longer incubation times with PEG and second antibody did not change the standard curve. During three months, the mean trace binding for AVP was 36.4% (SD 10.8%, n = 7) and 36.7% (SD 7.9%, n = 8) for ANP. A standard curve for AVP had the 50% intercept (ED\(_{50}\)) at 1.5
pg per tube or 4.6 pg/mL \((n = 8)\) (Figure 1). The 50% intercept for ANP is 7.2 pg per tube or 22.2 pg/mL (Figure 1). Addition of 1.6 pg of AVP, approximately equal to the concentration of \(1^{25}I\)-labeled AVP used in the assay, resulted in a 40% decrease in the percentage bound. Addition of 6 pg of ANP, approximately equal to the concentration of \(1^{25}I\)-labeled ANP used, resulted in a 35% reduction in the percentage bound. The smallest concentration of ANP that can be statistically distinguished from zero at 2 SD is 0.39 pg per tube; for AVP it is 0.13 pg per tube. The intra-assay CV of variation for AVP was 4.3%; for ANP, 3.8%. The interassay CV for AVP was 7.6%; for ANP, 6.7% (obtained from four different assays of four different plasma samples).

Antibody and antigen storage and use: For storage, AVP antibody was diluted fivefold more (5 mL) than the supplier recommends (1 mL). For assay, this stock solution was diluted a further twofold (1:20, 100 mL). ANP antibody was diluted 2.5-fold more (62.5 mL) than the supplier recommends (25 mL). For assay, this stock solution was diluted a further twofold (125 mL). The radiolabeled ANP and AVP were diluted with RIA buffer to 10 mL, quantified against RIA reference source for simulated \(1^{25}I\) (0.01 \(\mu\)Ci; NEN, Billerica, MA), and stored in 1-mL aliquots. For RIA, radiolabeled AVP and ANP were further diluted to give working concentrations (see RIA, above). Previous investigators noted stability problems with antibodies for ANP (12). We stored our antibodies at \(-80^\circ C\) and saw no changes during three months.

Physiological studies: Water loading produced a mean drop in \(P_{\text{osm}}\) from 292 ± 3.2 to 283 ± 2.3 mOsm/kg. Plasma AVP increased from 2.6 ± 0.32 to 1.42 ± 0.32 pg/mL \((P < 0.01)\) and plasma ANP decreased from 15.78 ± 1.6 to 10.38 ± 0.86 pg/mL \((P < 0.03)\). With water deprivation, \(P_{\text{osm}}\) increased from 292 ± 3.2 to 301 ± 3.7 mOsm/kg. Plasma AVP increased from 2.6 ± 0.32 to 4.07 ± 0.47 pg/mL \((P < 0.01)\) while plasma ANP increased from 15.78 ± 1.6 to 22.07 ± 1.73 pg/mL. The coefficient of correlation \((r)\) between \(P_{\text{osm}}\) and plasma AVP was 0.82 \((P < 0.0001)\); that between \(P_{\text{osm}}\) and plasma ANP was 0.88 \((P < 0.0001)\). Both of the patients with neurogenic diabetes insipidus had plasma AVP concentrations below the limit of detection for the assay \(< 0.15 \text{ pg/mL}\).

Discussion

Development of a simple, clinically useful RIA for AVP and ANP has proven to be more difficult than for many other polypeptide hormones. Limited specificity is inherent in the methods using direct RIA for ANP and AVP in plasma as reported by several authors (8, 10, 13, 14, 16). For indirect assays, the complexity of the extraction procedures and the lack of sensitivity of commercially available antibodies have limited the use of these assays in clinical chemistry laboratories. Further, the extraction methods for AVP are accompanied by poor and variable recoveries (17-20).

The method reported here is simple, sensitive, and reproducible for measuring AVP and ANP in plasma. One rapidly drying solvent system is used for simultaneous extraction of AVP and ANP. The extracts are lyophilized in an ordinary centrifuge under reduced pressure with no need for freezing in liquid nitrogen (12). One buffer system is used for both RIA dilutions, and the antibodies are commercially available.

Methods for extracting AVP from plasma have included the use of acetone and petroleum ether (7, 17), cold ethanol (19), or a solid support (21). For ANP, a gel-filtration method has been used (11). We tried to extract AVP and ANP by these methods and found the recoveries to be variable and nonlinear in the physiological range. Extraction with Sep-Pak C18 is a more selective method for isolating AVP and ANP and does not affect the stability of ANP as reported (16). However, heparinized samples tend to clog the cartridge. Acidification and centrifugation of plasma removes most of the precipitates, enhances sample flow, and improves recovery. Occasionally, high blank values were obtained for both AVP and ANP when Sep-Pak cartridges older than eight months were used. This was avoided by washing the column with 5 mL of tetrahydrofuran, then 50 mL of methanol, and storing the cartridge in methanol between uses. Acidification of plasma to pH 5.5 enhanced the stability of the C18 silica matrix, giving lower blank values and enabling us to re-use the cartridges two or three times. The solvent pair we used extracts AVP and ANP with high recoveries and has low vapor pressure for rapid drying of the samples.

AVP is not stable in plasma unless protease inhibitor PMSF and EDTA are added to the plasma; AVP is more stable (16). In keeping with this, the outdated plasma from blood bank that we used had very low ANP concentrations, but AVP values were within the normal range (Table 1). ANP or AVP standards added to this plasma showed excellent recoveries at both high and low concentrations (Table 1).

To improve assay sensitivity, we used nonequilibrium conditions and different dilutions of antiserum with different quantities of tracer for both ANP and AVP RIAs. Good sensitivity was obtained by incubating extracted plasma with antibodies for 16 to 18 h before incubating with radiolabeled antigens for an additional 16 to 18 h. Reproducible standard curves indicated that the concentrations of PEG and human gamma globulin we used were optimal to stabilize the precipitate formed, avoiding any reversal of the precipitation reactions. The use of human gamma globulin for immunoprecipitation has the advantage of being free of proteases that are present in the rabbit plasma that is used by some investigators (7, 10, 13, 16). During incubation, protease-induced damage was further minimized by using BSA that had been treated with PMSF and heat inactivation. This is especially important, owing to the long incubation periods used in RIAs. The nonspecific binding for our method gave lower values than do other methods in which charcoal-dextran or second antibody precipitation is used (9, 11, 12, 15).

During water loading and dehydration, plasma AVP and ANP concentrations generally conformed to the expected physiological relationship (15, 18). Further, in plasma of patients with neurogenic diabetes insipidus, AVP was undetectable \(< 0.13 \text{ pg/mL}\). Therefore, the present assay is both sensitive and specific for AVP and ANP in plasma and will enable a clinical chemist to successfully test the various forms of polyuria.

Supported by R01 HL 34837 and R01 HL 39165 from the Heart, Lung, and Blood Institute, N.I.H. C.R.B. is an Established Investigator of the American Heart Association. We thank Karen Humphrey for secretarial help.

References

Identification of HIV-Specific Oligoclonal Immunoglobulins in Serum of Carriers of HIV Antibody

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Zone electrophoresis on agarose gel was performed on serum samples from HIV-antibody carriers and negative controls. Nitrocellulose strips precoated with an HIV preparation were then placed on top of the gels and developed by an immunoblotting procedure. A positive reaction was demonstrated between the HIV antigens and the HIV-antibody-positive serum samples with hypergammaglobulinemia and oligoclonal IgG bands. A negative reaction was found between the HIV antigens and HIV-antibody-negative serum samples from a normal person and a patient with monoclonal gammopathy. The presence of oligoclonal IgG bands in the serum of HIV-antibody carriers, and their positive identification with HIV antigens, indicates a specific immune response of the host to the HIV infection and supports the use of oligoclonal IgG bands as markers to follow the course of HIV infection.

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Received December 3, 1987; accepted February 4, 1988.

The phenomenon of oligoclonal banding is a new development in zone electrophoresis of serum proteins. Using a sensitive electrophoretic technique (1), we reported a high incidence of oligoclonal immunoglobulin bands (OIB) in serum samples of patients with acquired immunodeficiency syndrome (AIDS) (2). Presence of paraproteins in sera of AIDS patients has been reported (3, 4). Also, we found OIB in serum samples from asymptomatic carriers of human immunodeficiency virus (HIV) antibody. The OIB were identified as immunoglobulin G (IgG) heavy chain only and mixed κ and λ light chains (5). In this report we present evidence of a positive reaction between OIB of serum samples from HIV-antibody carriers and HIV antigens. In contrast, no reaction was observed between the HIV antigens and antibody-negative serum samples from a normal person and a patient with monoclonal gammopathy.

Materials and Methods

Electrophoresis. In this study we used serum samples from asymptomatic persons who tested positive for HIV antibodies, first with an enzyme-linked immunosorbent assay (ELISA) and then with the Western blot test (Biotech Research Laboratories, Rockville, MD, 20857). We also used serum samples from a healthy person and a patient with a