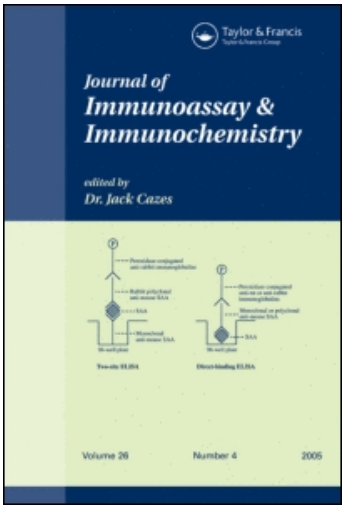

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DEVELOPMENT OF A SOLID-PHASE RADIOIMMUNOASSAY FOR THE DETERMINATION OF ARGININE-VASOPRESSIN IN URINE

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(KEY WORDS: arginine-vasopressin; solid-phase radioimmunoassays; hormone assays; neuropeptides; peptide hormones; urine extracts).

ABSTRACT

A new solid-phase radioimmunoassay (RIA) has been developed for measuring arginine-vasopressin (AVP) in urine. AVP is first extracted from urine by adsorption on Vycor glass powder and eluted with acetone-water (60:40). The mean recovery is 75.3 ± 2.2% (n = 18). The organic extract is evaporated to dryness and reconstituted in the assay buffer. Aliquots of this extract are then incubated with ^{125}I-AVP in polystyrene LKB tubes previously coated with the antiserum (1:50000) for 48 hours. The free radioactive fraction is removed by aspiration and the tubes are counted. Values correlate well with those obtained by liquid-phase RIA using dextran-charcoal. Urinary AVP concentrations in normal Sprague-Dawley rats and rats with varying degrees of hydration have been measured.

INTRODUCTION

To explore the role of antidiuretic hormone (AVP) in physiological and pathological states, numerous assays for its determination in biological fluids have been developed. Application

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of the RIA techniques for AVP is associated with difficulties related to the low plasma concentration of AVP, to the presence of interfering substances in urine (1) and in plasma (2), to the development of antibodies of high affinity and sufficient specificity toward this small cyclic polypeptide.

There is considerable variation in the methodology, particularly in the extraction of the samples in order to concentrate the hormone and to overcome the effects of non-specific interfering substances. The extraction of AVP has been carried out by adsorption on Florisil (3,4,5), bentonite (6), Fuller's earth (7) and Vycor (8,9). Cation exchange resin (Isorex) has been also used (10). More recently it has been proposed to extract this peptide on octadecasilyl-silica (C_{18} Sep-Pak) (11,12). Another method for removing the interfering components is extraction with acetone-ether (13,14). Despite the fact that the immunogenic portion of this peptide is known to be localized in the ring moiety (particularly the phenylalanyl in position 3), the production of antibodies which are specific and which have a high affinity for this hormone is difficult (15). Most of the RIA procedures use non-equilibrium conditions requiring a 3 to 7 days incubation time followed by a separation of the bound fraction from the free by double-antibody or dextran-charcoal precipitation techniques.

The present report describes the development of a simple and rapid solid-phase AVP RIA procedure for the measurement of this

hormone in urine extracts and its comparison to the results of classical RIA.

MATERIALS AND METHODS

Materials

Vycor glass powder (120 mesh) was obtained from Corning Glass Works, Corning, NY; Dextran T-70 from Pharmacia Fine Chemicals, Uppsala, Sweden; activated charcoal (Norit-A), sodium azide and methanol HPLC grade from Fisher Scientific, Fair Lawn, NJ; tetrahydrofuran from Baker Chemical, Phillipsburg, NJ; Sep-Pak from Waters Associates Inc., Milford, MA; and bovine serum albumin (BSA) Fraction V (Lot no. 362) from Miles Laboratories, Elkhart, IN.

Synthetic AVP was purchased from Ferring AB (Malmö, Sweden) with a biological activity of 400 I.U. /mg (Lot no. BAA207). Synthetic arginine-vasotocin (AVT), lysine-vasopressin (LVP) and oxytocin (OT) were obtained from Calbiochem-Behring Corp., La Jolla.. Monoiodinated ^{125}I-AVP (2200 Ci / mmol) was purchased from New England Nuclear, Boston, MA (NEX-128). For the recovery study, ^{3}H-AVP (51 ci / mmol) was purchased from the same company (NET-800). The commercial rabbit antiserum against arginine-vasopressin was obtained from Calbiochem-Behring (cat. no. 969115 and lot no. 293041). All other chemicals and buffer salts were of reagent grade.

Procedures

Collection of Samples

Urine was collected in polypropylene centrifuge tubes (50 ml). Twenty µl of a 50% solution of trifluoroacetic acid in water was added per ml of urine to acidify the sample to pH 4-4.5. The samples were immediately frozen and stored at -20°C; they were extracted and assayed after a period not exceeding two weeks. Samples could also be stored frozen at -20°C, pH 4, for up to three months without any observed loss of immunoreactive AVP.

Extraction of AVP from urinary samples

Urine was thawed at 25°C. Before extraction, 20 µl of a BSA solution (50 g/L) per ml of urine was added to prevent the adsorption of AVP. AVP was extracted from the urine with Vycor glass powder that had been washed with distilled water and purified acetone. The powder was thermally activated by heating it at 700°C for 12 h, and kept at 100°C until used. To each 1 ml sample, 50 mg of cooled Vycor glass was added in 1 ml of distilled water; the mixture was then rotated in the vertical plane for 30 min at 4°C. After a 2 min centrifugation (2000 rpm) the supernatant was aspirated and the glass powder was washed, first with 3 ml of distilled water and afterwards with 2 ml of 1 mol/L HCl. The adsorbed hormone was eluted from the glass with 1 ml of acetone/water (60/40 V/V) during a 30 min rotation period at

4°C. The eluate containing AVP was carefully collected in a polypropylene tube by aspiration with a micropipet and evaporated in a rotary evaporator (Speed Vac Concentrator Model SVC-100H, Savant Instruments, Hicksville, NY).

For the classical liquid-phase RIA, the extraction method was different. Small columns packed with octadecasilyl-silica (C_{18} Sep-Paks) were used. Each column was activated with 5 ml of tetrahydrofuran. The column was then washed with 10 ml of distilled water followed by 10 ml of a 20 mmol/L triethylamine acetate (TEA) solution pH 4. Each 1 ml of the urine sample was loaded into a syringe and pushed slowly through the column over a period of 1 min. The column was then rinsed with 10 ml of TEA solution, and the peptide eluted with 3 ml of pure methanol. The eluate was collected into a polypropylene tube and evaporated in the same way as with the Vycor extraction. The dry extracts were used for RIA.

Antiserum Preparation

The immunogen was prepared by conjugating 4 mg of AVP to 20 mg of thyroglobulin in presence of 1 mg of carbodiimide (16). The incorporation yield was 40% as measured with iodinated AVP. Female New Zealand white rabbits received a primary immunization of thyroglobulin-vasopressin conjugate (125 μg) in 0.9% saline (1 ml) emulsified with Freund's complete adjuvant (1 ml) as multiple dorsal subcutaneous injections. At intervals of 1 and 2

months following the primary immunization, booster injections of immunogen were administered the same way. Two weeks after booster injections, blood samples were collected from the central ear artery and plasma was separated and stored at -70°C.

Preparation of coated tubes

The coated tubes were prepared as described elsewhere (17, 18). Briefly, 0.9 ml of antiserum diluted 1:50000 in 0.1 mol/L sodium carbonate buffer, pH 9.5 was added with an automatic pipettor to each polystyrene LKB tube. The coating of antibody was allowed to proceed for 20 h at 4°C. The antiserum was removed by suction and the tubes were rinsed once with 2 ml of the rinsing buffer which consisted of 10 mmol/L potassium phosphate buffer, pH 7.4, 9g/L NaCl and 1 g/L BSA. The tubes containing 2 ml of rinsing buffer were stored at 4°C for a period not exceeding 1 month.

Radioimmunoassay

All reagents were diluted with the assay buffer which consisted of 50 mmol/L potassium phosphate buffer, pH 7.4, 1 g/L BSA and 0.2 g/L sodium azide. For the standard curve, the stock solution of synthetic AVP (100 µg/ml) in 2 mol/L acetic acid was used for serial dilutions with the assay buffer to final concentrations ranging from 7.8 to 2000 pg/ml. For sample analysis, the dry extractswere reconstituted in 0.5 ml of assay buffer

and sonicated (Bransonic bath model 32) for 2 min to facilitate the solubilization of the residue. Quadruplicate 100 µl aliquots were taken from each reconstituted sample for RIA. After careful removal of rinsing buffer, the reactants were added to antibody-coated tubes in the following sequence: 100 µl of AVP standard dilution or urine extract, 100 µl of radioactive vasopressin (2500 cpm) made up to 900 µl with the assay buffer. A reference tube (without unlabeled hormone) and nonspecific binding tube (without either unlabeled hormone and antibody) were also processed. All points of the standard curve were established in duplicate. The tubes were mixed on a LKB mixer and incubated for 48 h at 4°C. The reactants were carefully removed by suction; the tubes were then rinsed with 2 ml of rinsing buffer. Radioactivity bound to the antibody was counted for 5 min in a LKB 1270 Rackgramma II counter. Raw counter data was entered and stored in a computer file for processing with a computer program (19). Data were expressed as bound fraction counts versus logarithm of AVP concentration in a sigmoid standard curve. Different volumes of extracts of AVP from urine were tested against the standard curves for parallelism.

The liquid-phase RIA was performed using a commercially available antiserum (Calbiochem-Behring). The final dilution in assay buffer was 1:320000. The quoted cross-reactivity with OT and AVT was less than 1% and less than 0.2% with LVP. The entire procedure of the RIA was performed at 4°C in polystyrene

tubes. Reagents used were the same as used with the solid-phase RIA except for the 100 µl of antiserum dilution in assay buffer. The total volume of incubation was 800 µl. A buffer blank was used to determine the nonspecific binding and to test the charcoal separation system. After vortexing, the tubes were incubated for 48 h at 4°C. Separation of antibody-bound from free ^{125}I-AVP was performed by adding of 1 ml of dextran-charcoal suspension (6.25 g/L charcoal and 0.625 g/L dextran in 50 mmol/L potassium phosphate buffer, pH 7.4) with constant stirring. After agitation, the tubes were immediately centrifuged at 3000 g for 10 min at 4°C. The supernatant fluid was promptly decanted and its radioactivity counted in a gamma counter.

Antiserum Specificity

To determine the specificity of the antiserum, the AVP standard was replaced by increasing concentrations of structurally related peptides such as OT, AVT and LVP. The cross-reactivity of each related peptide was defined as the ratio of ED_{50} for AVP to ED_{50} for the substance studied; ED_{50} is the molar concentration for each respective peptide required to inhibit 50% of the binding of radioligand to the antiserum (20).

Quality Control

One human urine sample (stored in 3 ml aliquots at -20°C) was extracted and assayed in duplicate in 9 different assays to

evaluate the within-assay and between-assay coefficients of variation (21).

Animal Studies

A group of six male Sprague-Dawley rats were maintained without water for 24 hours after a control period of 24 hours during which they had access to water ad libitum. Another group of five rats received an oral water load of 20 ml/kg every 2 hours for 8 hours after a control period of 8 hours. The rats were placed into metabolic cages and their urine collected for the periods mentioned.

Statistics

Statistical analyses were performed by using an analysis of variance (ANOVA) with an SPSS software (22). A Bartlett test for homogeneity of variance was performed for the comparisons of the independent groups. The values were expressed as mean ± SEM.

RESULTS

Characteristics of Antiserum 93

Within two months, two out of four rabbits immunized produced antisera with titers which increased in time. Two weeks after each immunization the titer was determined by doing successive dilutions of the antiserum. The highest titer elicited was obtained from rabbit 93 and was selected for further studies. The antiserum titer used in the RIA was 1: 50000 and

gave a binding of 30 to 40% of the total radioligand added. Prolonging the incubation beyond 48 hours did not increase further the binding of the radioligand. The cross-reactivity on a weight basis was 0% with OT and less than 2% with LVP and AVT. Scatchard analysis (23) showed the presence of a homogeneous antibody population. The 1:50000 dilution of antiserum used in the solid-phase assay was characterized by a binding capacity of 5.79 pM and an affinity constant (k_1) of 0.122 pM^{-1}.

Standard Curve

A typical standard curve with 95% confidence limits is shown in figure 1. Statistical analysis of standard curves showed a detection limit of 1.5 pg/tube. The detection limit was considered as the minimum amount of AVP that could be statistically distinguished from zero at two standard deviations. The standard curves were reproducible: analysis of ten standard curves (logit-log linear regression) showed a slope of -0.92 ± 0.02 (mean ± SE) and ED_{50} of 23.7 ± 1.2 pg/tube (mean ± SE). The nonspecific binding of ^{125}I-AVP to polystyrene tubes was included in the range of 1.00 to 1.50%.

Accuracy and Precision

In the recovery studies, different quantities of synthetic AVP were added to a rabbit sample with low AVP (50-125 pg/ml). The mean recovery (± SE) was 75.3 ± 2.2% (n=18). A slightly higher recovery was observed using ^{125}I-AVP [86.6 ± 0.9% (n = 8)]

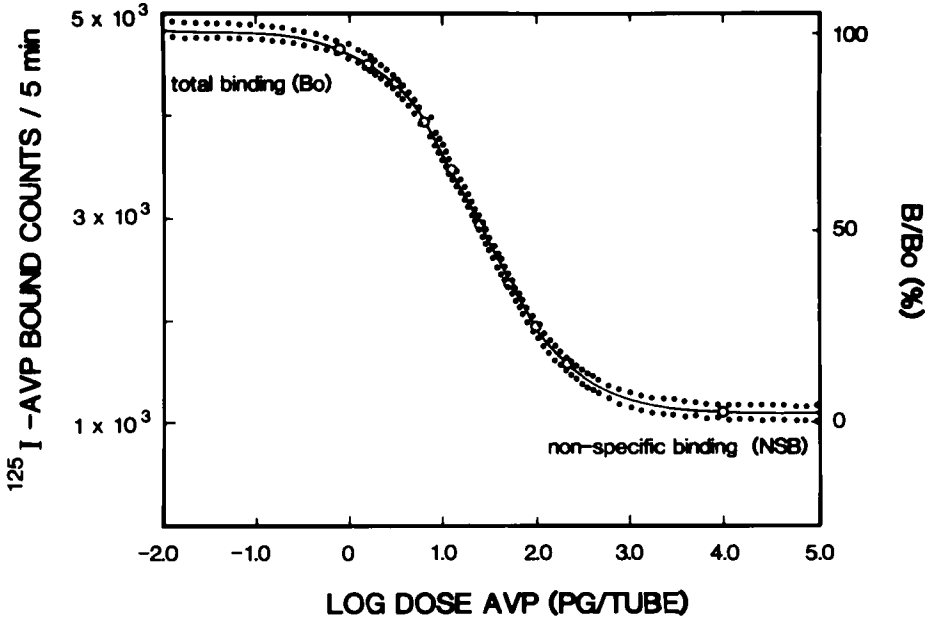

Figure 1: RIA standard curve for AVP (^{125}I-AVP bound versus log AVP).
B is the amount of labeled hormone bound to the antibody in the presence of unlabeled hormone. B_0 is the amount of labeled hormone bound to the antibody in the absence of unlabeled hormone. The non-specific binding is defined by the binding of ^{125}I-AVP in absence of the antibody. Radioactivity bound (-), mean ± 2 SD (...).

or ^{3}H-AVP [87.9 ± 1.4% (n = 4)]. Urine concentrations were not corrected for recovery. The within-assay CV was 6.3% (n = 9) and the between-assay CV 10.9% (n = 9). The mean concentration (± SE) of AVP in human urine assayed for quality control was 197.2 ± 5.8 pg/ml (n = 18). The results from solid-phase RIA were validated by comparing them to those obtained by classical liquid-phase RIA. Rat urines containing 30 to 100 pg/ml of AVP

TABLE I

Comparison of rat urinary AVP concentrations obtained by solid-phase and liquid-phase RIA[a]

METHOD	AVP (pg/ml) URINE #1	URINE #2	URINE #3
Solid-phase RIA	27.08 ± 2.59 (N = 13)	72.53 ± 6.22 (N = 13)	81.09 ± 3.69 (N = 6)
Liquid-phase RIA	[b]33.36 ± 3.03 (N = 12)	[b]74.84 ± 2.39 (N = 11)	[b]96.82 ± 7.23 (N = 4)

[a] Aliquots (1 ml) of acidified rat urines were extracted and assayed according to the two RIA procedures as described in Material and methods.

[b] No statistically significative difference (Anova at $p > 0.05$)

were extracted and assayed according to solid-phase and liquid-phase methods described before. The urinary AVP concentrations determined by both assays are shown in Table 1. There was no statistically significant difference in the urine AVP concentrations obtained by the two different methods.

Parallelism

Human urine was extracted six times with Vycor glass powder as described in procedures and the dried extract was redissolved

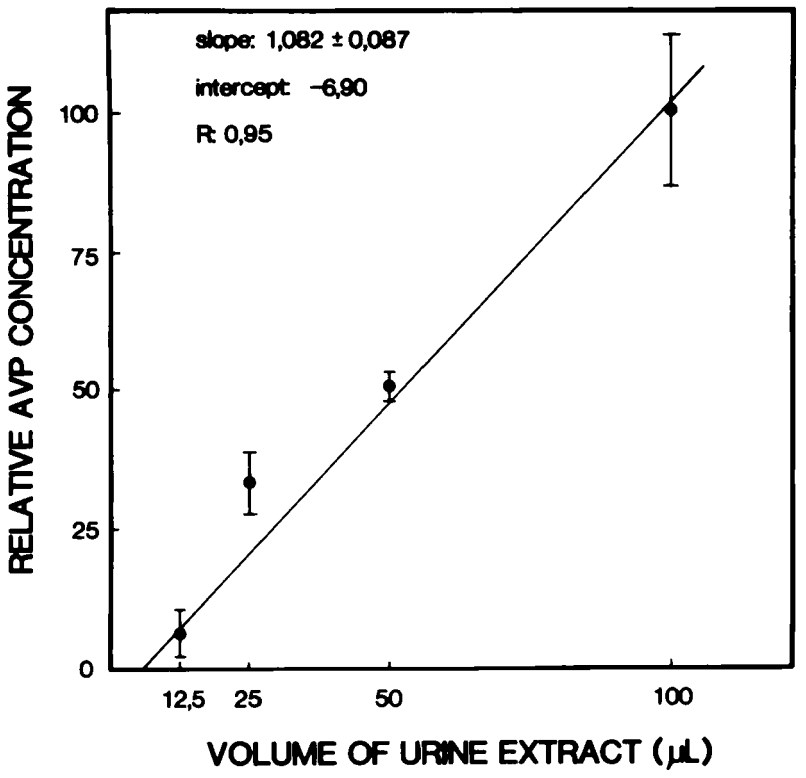

Figure 2: Parallelism testing of human urine extracted with Vycor glass powder.
Different volumes of extracts of human urine were assayed according to the solid-phase RIA procedure. The relative AVP concentration of 100 corresponds to 100 µl of urine extract containing 73 pg/ml of AVP. Values are mean ± SEM.

in 500 µl of assay buffer. Extract volumes ranging from 12.5 to 100 µl were used for the solid-phase RIA. As shown in figure 2 there was a linear response between the volume of extract assayed and AVP content measured.

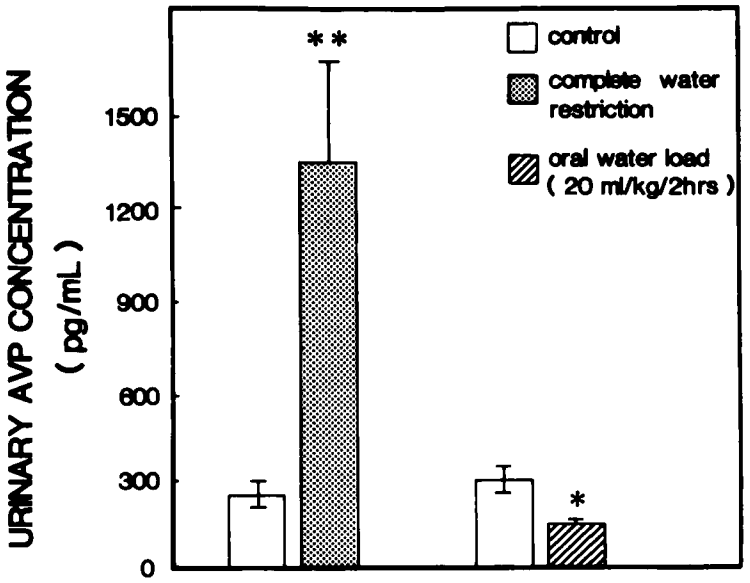

Figure 3: Complete water restriction and oral water load effects on urinary AVP concentration.
Male Sprague-Dawley rats (n=11), following a control period were divided in 2 groups: group 1 (n=6) are under complete water restriction for 24 hours; group 2 (n=5) received an oral water load (20 ml/kg every 2 hours for 8 hours). Urines were then collected and assayed by solid-phase RIA procedure. Values are mean ± SEM. Asterisks show significant differences from the respective controls.* p=0.001; ** p=0.005.

Animal Studies

As shown in figure 3, the urinary AVP concentration was 310 ± 41 pg ml (n=6) in normal control rats and 154 ± 18 pg/ml in the same rats after water-loading. The urinary AVP concentrations were significantly lower (p=0.005) in water-loaded rats. In a second group of rats, the average urinary AVP concentration was 250 ± 45 pg/ml (n=5) in the control study and 1318 ± 147 pg/ml in the same rats after complete fluid restric-

tion. The urinary AVP concentrations were significantly higher ($p<0.001$) in water-deprived rats than in control rats.

DISCUSSION

The present study was performed in order to develop an RIA for AVP in urine using solid-phase adsorption of the antibody. The Vycor extraction was chosen because it eliminates salts and urea which could have an unexpected influence on the binding of AVP to the antibody. This procedure allowed also a selective separation of this peptide from the endogenous interfering substances, particularly urinary pigments which could affect the integrity of the antibody coating onto the wall of the tubes during the incubation period. The recovery of AVP was 75.3 ± 2.2% and was comparable to recoveries encountered in the literature which vary from 50 to almost 100% (1-14). Dogterom et al. (8) and Landgraf (9) obtained recoveries of AVP of 69.4 ± 6.5% and 84.4 ± 32.7% respectively when extracting AVP from plasma using Vycor glass powder.

The adsorption of antibody to polystyrene tubes is simple and rapid. In less than 24 hours it is possible to prepare several hundred tubes. An LKB automatic pipettor can be used to pipet the 900 µl of antibody dilution in each plastic tube. It is then possible to prepare stocks of tubes sufficient for one month's assays.

The standard curves were highly reproducible and thus permitted urinary AVP determinations after a single incubation

of 48 hours. This is advantageous when compared to assays requiring 3 to 7 days of incubation. Under our experimental conditions, the detection limit was 1 to 1.5 pg/tube, which is well below the sensitivity needed to measure urinary AVP levels in human or animals. The within- and between-assay CV are both below 11% which is a good indication of the stability of the assay. Most RIAs published for AVP in plasma and urine have a within-and between-assay CV between 10 and 20%. The low CV with the solid-phase technique could be explained at least by the simplicity of the separation of bound from free. This is an advantage compared to the dextran-charcoal or double-antibody techniques. Furthermore, the latter techniques depend on the quality of the charcoal or the second antibody used. The good correlation between our new solid-phase method and a classical method using dextran-charcoal validates the results obtained with our RIA. The decreased urinary AVP levels in water-loaded rats versus controls and the increased urinary AVP levels in water-deprived rats (figure 3) versus controls demonstrate the ability of the assay to measure physiologic changes in AVP secretion in response to changes in plasma osmolality (24).

Our assay compares favorably with the other radioimmunoassays for AVP described (1-14), although less sensitive than the assay described by Robertson et al. (14). This new solid-phase method could be particularly useful for rapid routine

measurement of AVP in physiological studies of secretion of this hormone from pituitary posterior lobe, or its urinary excretion.

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