Radioimmunoassay of Arginine Vasopressin in Human Plasma and Urine, a Resin Microcolumn Method

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KIMURA, T., MATSUI, K., OTA, K. and YOSHINAGA, K. Radioimmunoassay of Arginine Vasopressin in Human Plasma and Urine, a Resin Microcolumn Method. Tohoku J. exp. Med., 1980, 131 (1), 37-46 — A new method was developed for the estimation of arginine vasopressin (AVP) in plasma and urine. Samples were extracted by a microcolumn of resin and assayed radioimmunologically using a highly sensitive antiserum to AVP. Ion-exchange resin, CG-50, H+ form, packed in a small column (diameter 4 mm, height 6 mm), was proved effective to remove the interfering substances and to concentrate the AVP in the sample. The application of 80% acid acetone successive to diluted HCl brought about a consistent recovery of AVP from the resin column. Recoveries were 66.4±8.5% for plasma and 85.4±9.7% for urine. In normal subjects plasma AVP levels were 3.9±0.3 pg/ml (mean±s.d.) in ambulatory states, 4.9±0.6 after overnight fast, and 0.4±0.2 after water loading. High levels of 2.0-24.2 pg/ml were obtained in patients with syndrome of inappropriate secretion of ADH (SIADH), low values of 0-1.8 pg/ml in diabetes insipidus. Urinary excretions of AVP were 117.4±59.4 ng/24 hr (mean±s.d.) in normal controls, 191±177.0/24 hr in SIADH, and 17.0±12.0/24 hr in diabetes insipidus. — antidiuretic hormone; arginine vasopressin; radioimmunoassay of arginine vasopressin; diabetes insipidus; syndrome of inappropriate secretion of ADH

Recently, plasma and urinary levels of arginine vasopressin (AVP), the antidiuretic hormone (ADH), have been successfully measured by radioimmunoassay. Most of these assay systems necessitate some preliminary steps to extract the hormone from the specimens (Miller and Moses 1972; Husain et al. 1973; Robertson et al. 1973; Fressinaud et al. 1974; Skowsky et al. 1974; Beadwell et al. 1975; Merkelbach et al. 1975; Morton et al. 1975; Shade and Share 1975; Shimamoto et al. 1976; Baylis and Heath 1977; Keil and Severs 1977), except the one reported by Fyhrquist et al. (1976), because the concentrations of AVP are extremely low and there are many factors in plasma and urine which may interfere with the radioimmunoassay. However, even if using these extraction techniques, consistent results could not always be obtained in every laboratory. These inconsistencies may

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largely be due to the differences in the extraction procedures or in the specificity of AVP antisera used in each assay.

Of a variety of the extraction procedures, a resin column chromatography has been used widely in the bioassay of AVP (Yoshida et al. 1963; Moran et al. 1964; Claybaugh and Share 1972; Kimura et al. 1974), but this technique has not been applied so frequently in the radioimmunoassay. The resin column method required a large amount of solution to elute AVP from the resin and a lot of time to concentrate the eluate for the assay.

This paper aimed to report a sensitive and reliable radioimmunoassay of plasma and urinary AVP, employing both a new extraction procedures through a resin microcolumn chromatography and a highly sensitive antisera developed against AVP.

MATERIALS AND METHODS

Antiserum production. Three mg of synthetic arginine vasopressin (Sigma Co, 367 IU/mg) were conjugated with 30 mg of bovine serum albumin by 0.02 M glutaraldehyde. After dialysis and lyophilization, conjugated vasopressin, dissolved in 0.9% saline, was emulsified with an equal volume of complete Freund's Adjuvant (Difco Laboratories) and injected intradermally into 8–10 sites in male albino rabbits at one week's intervals for 5 months. Each injection contained 0.1 mg of conjugated vasopressin.

AVP iodination. Iodination of synthetic AVP was performed by the chloramine T method originally described by Greenwood et al. (1964). One μg of AVP was allowed to react with 1.0 μCi of 125I in the presence of chloramine T. The reaction mixture was applied to a superfine Sephadex G-25 column for purification and eluted with 0.01 M acetic acid containing 0.5% BSA.

Radioimmunoassay. The assay was performed in a non-equilibrium system; plasma and urine extracts and various amounts of unlabeled AVP were placed in each tube and an appropriate amount of diluent (0.05 M phosphate buffer, pH 7.4, containing 10^{-4} M EDTA, 2×10^{-4} M L-cystine, 0.1 mM Neomycin sulfate (Upjohn Co.), 10 mM epsilon amino caproic acid, 5 ml/liter normal rabbit serum and 1.25 mg/ml BSA) was added to yield a final volume of 200 μl. Fifty μl of the antiserum, diluted 6×10^{4} with the diluent described above, was added to each tube. The tubes were incubated at 4°C for 24 hr, and then approximately 1500 cpm of 125I-AVP dissolved in 50 μl of the diluent was added. They were incubated at 4°C further for 3 days. The assay was performed in duplicate.

The bound AVP was separated from free by the polyethylene glycol (PEG) method (Husain et al. 1973; Robertson et al. 1973); 100 μl of 1% solution of bovine gamma globulin (Fraction II, Schwarzmann) were added to each tube followed by 1 ml of 25% solution of cold PEG. The tubes were mixed on a vortex mixer and centrifuged at 3000 rpm at 4°C for 45 min, the precipitate was counted for 10 min in an automatic gamma counter. The counts of the precipitate of labeled AVP were expressed as percent of the control tubes.

Extraction

Blood. Five ml of heparinized venous blood were drawn and immediately centrifuged at 3000 rpm for 20 min at 4°C. The separated plasma was frozen immediately at −20°C and AVP was usually extracted within 1 week after collection.

Two ml of plasma were added to 4 ml of 10% trichloroacetic acid (TCA). After vortex mixing for 1 min, the mixture was centrifuged at 3000 rpm for 15 min, the supernatant was saved. The precipitate was extracted again with 4 ml of 10% TCA. The supernatants were combined, and washed three times with an equal volume of diethyl ether. The pH of aqueous phase was then adjusted to 4.5 with diluted ammonia, and the sample was applied onto a CG-50 resin microcolumn, H+ form, 6 mm in height and 4 mm in
diameter. The flow rate was kept at less than 250 μl/min. The column was then washed with 2 ml of distilled water followed by 500 μl of diluted HCl (pH 2.0). AVP was subsequently eluted with 2 ml of 80% acid acetone (pH 1.5, acidified by HCl). The eluate was washed with 2 ml of diethyl ether, and was dried under gentle air current. The dried material was dissolved in 0.5 ml of the diluent. Two 200 μl aliquots were then assayed.

Urine. Urine was kept at 4°C in a bottle containing 2 ml of gracial acetic acid until brought to the laboratory. They were then stored frozen at −20°C until assay.

AVP was extracted from 2 ml aliquots of urine. The urine was adjusted to pH 4.5 with ammonia as mentioned above and then charged on the resin column. The extraction procedures were the same as those of plasma AVP except for using 3 ml of 80% acid acetone for elution.

Experimental protocol. Blood and urine samples were collected from normal subjects, patients with diabetes insipidus and patients with syndrome of inappropriate secretion of ADH (SIADH) under variously hydrated states. (1) Normal hydration: Blood was collected from 30 normal subjects and 7 patients with SIADH 2 hr after meal in the ambulatory states. Urine samples were taken from 10 normal subjects, 9 patients with diabetes insipidus and 9 patients with SIADH for 24 hr under ambulatory states with no smoking. (2) Water deprivation: Blood was collected from 31 normal subjects early in the morning after overnight fasting. For patients with diabetes insipidus (n=11), dehydration test was done to about 3% reduction in the body weight, and blood was taken. To study the urinary AVP, 25 normal subjects were deprived of fluid for 14 hr overnight, then 1 hr-urine sample was taken. (3) Water loading: Blood was collected from 8 normal subjects 1-2 hr after an oral water load of 20 ml/kg body weight. Urinary samples were taken from 6 normal subjects after the water loading. Urine was discarded for the first 1 hr following the loading, but was collected at hourly intervals thereafter.

Plasma and urinary osmolalities were determined by an Advanced osmometer.

RESULTS

Antiserum. Three rabbits were immunized with AVP conjugate, and two of them developed antibody. After 5 months, one antiserum bound 50% of added labeled AVP at a final dilution of 1×10⁵ and the other 1.5×10⁵. The latter was used in the present study.

The antiserum was highly specific for AVP and showed no cross reactivity to deamino-D-arginine vasopressin and oxytocin, but showed a little cross reaction to lysine vasopressin (0.5%) as shown in Fig. 1. Immunized rabbits showed polydipsia and polyuria, indicating the development of diabetes insipidus caused by antibody to endogenous ADH.

Iodination of AVP. The iodination mixture gave three major peaks of radioactivity in the chromatography on Sephadex G-25. The first two peaks did not bind antibody significantly, and the second peak represented free ¹²⁵I. The binding of more than 90% with an excess antiserum was observed with the third peak. This labeled AVP was repurified by the Sephadex column before assay.

The specific activity thus calculated was 250 to 300 mCi/mg and the specific activity, estimated from assay of serial dilutions of labeled AVP, averaged 1200 to 1500 μCi/μg (1–1.5 pg/ml). Labeled AVP was stable for 2 months, when stored at −20°C in 0.1 M acetic acid.

Radioimmunoassay. Fig. 1 shows a series of dose-response curve of vasopressin. The curves obtained with the extracts of charcoal-treated plasma (vasopressin free
plasma), to which various amounts of AVP had been added, paralleled to those of synthetic AVP dissolved in the diluent. It was also essentially the same as those obtained with plasma from patients with diabetes insipidus. No significant differences was noted between the charcoal-treated plasma and the plasma from patients with severe diabetes insipidus. Therefore, they were used as a plasma blank in this assay system.

The dose-response curves obtained with the extracts of AVP free urine to which various amounts of AVP had been added were also similar to those of pure AVP solution. Urine blank showed about 15% depression in binding. Urine samples taken from an adult volunteer undergoing maximal water diuresis were used in this experiment as vasopressin free urine.

The limit of detection of the standard curve was 0.4 pg/tube of synthetic AVP.

Recovery and sensitivity. The recoveries of added AVP in plasma were as follows; in the range of 1.6 to 6.4 pg/ml of plasma, 67.0±9.1% (mean±s.d., n=29), at 12.8 to 51.8 pg/ml, 65.7±8.2% (n=34), all recoveries combined, 66.4±8.5% (n=64). The recoveries of added AVP in urine were as follows; at 1.6 to 6.4 pg/ml of urine, 87.3±9.3% (n=22), at 12.8 to 102.4 pg/ml of urine, 85.6±9.9% (n=28), all recoveries combined, 85.9±9.7% (n=50).

Pooled plasma or urine with added AVP to give concentrations of 3.2 pg/ml or 25 pg/ml was assayed to test the reproducibility. Inter-assay coefficient of variation for plasma AVP was 12.8% (6 estimations), and the coefficient for urine was 11.8% (8 estimations).

Intra-assay coefficient of variation for plasma was 13.3% in 9 assays and the coefficient for urine was 10.2% in 6 assays.

Extraction procedure. Blood. When 2 ml of plasma were extracted twice with 4 ml of TCA, the recovery was 70% or higher. The recovery was not
increased when the TCA extraction was repeated more than twice.

The extracted sample was washed three times with 8 ml of ethyl ether. When the sample was applied on a resin column at the flow rate of 250 µl/min, labeled AVP was completely adsorbed on the resin.

No elution of AVP occurred when the column was washed with distilled water and diluted HCl (pH 2), but the AVP was quantitatively eluted with less than 2.0 ml of acidified 80% acetone. Its total recoveries were 96.4±1.0% (mean±s.D., n=5). When the elution was performed by acetone HCl alone, a large amount of eluate was required to achieve a complete elution of AVP from the column as shown in Fig. 2. Therefore, pretreatment with diluted HCl was an essential step to achieve a good recovery with a small amount of eluate.

Urine. Purification with TCA and ethyl ether was not required in the extraction of urinary AVP, but the other steps were the same as for the plasma AVP. But 1 ml of diluted HCl for pretreatment and 3 ml of 80% acid acetone for elution were found suitable for urine samples to get a good recovery.

**AVP levels in human plasma and urine.** Fig. 3 shows plasma AVP levels in

![Fig. 2. Comparison of the patterns of elution of labeled AVP from the resin column by 80% acetone HCl.
A: Elution without the pretreatment of dil. HCl.
B: Elution with the pretreatment of dil. HCl.

![Fig. 3. Plasma AVP levels in normal subjects, patients with SIADH (ectopic ADH producing tumor, *) and patients with diabetes insipidus.](image-url)
normal subjects and patients with SIADH or with diabetes insipidus under a variety of hydration. In normal subjects, AVP levels 2 hr after meal in the ambulatory states were 3.9±0.3 pg/ml (mean±s.d., n=30). Following an overnight fast, AVP levels were 4.9±0.6 pg/ml (n=31). After the water loading as described above, AVP levels were lowered to 0.4±0.2 pg/ml (n=8). In patients with SIADH, AVP levels were 2.0 to 24.2 pg/ml under ambulatory states. Patients with diabetes insipidus showed low AVP levels less than 1.8 pg/ml under dehydrated states.

Fig. 4 shows the relationship between plasma osmolality and plasma AVP levels under various states of hydration. No AVP was detected in blood in normal subjects, when plasma osmolality was less than 270 mOsm/kg, but there was no significant correlation between the plasma osmolality and AVP level. Patients with diabetes insipidus showed low levels of AVP despite high plasma osmolality and the reverse was observed in patients with SIADH.

Fig. 5 illustrates urinary excretion of AVP in normal subjects, patients with SIADH and those with diabetes insipidus under various states of hydration. In normal subjects, urinary excretion of AVP were 117.4±59.4 ng/24 hr (mean±s.d., n=10) under ambulatory states with no smoking. After overnight fast urinary AVP was 4.0±2.5 ng/hr (n=25). Following the water load, urinary AVP decreased to 0.6±0.4 (n=6). In patients with SIADH, urinary AVP was very high.
177.0 ng/day \( (n=9) \). In patients with diabetes insipidus, it was low, 17.0±12.0 ng/day \( (n=9) \).

Fig. 6 shows the relationship between urinary osmolality and hourly AVP excretion. Urinary AVP \( (y) \) increases with the rise in urinary osmolality \( (x) \), and the following relationship was obtained: \( y = 3.5 \log x - 5.9 \) \( (r=0.72, p<0.01, n=30) \).

**DISCUSSION**

Recent studies suggested that plasma AVP levels were extremely low compared with urinary AVP levels in various physiological conditions. The present assay system made it possible to measure accurately AVP concentrations of 0.4 pg/tube or less. Thus, when 2.0 ml of plasma or urine specimens were applied on the assay, these sensitivities are obviously sufficient to assess the various pathophysiological changes of AVP in these specimens.

The existence of factors in plasma and urine which strongly interfere with the radioimmunoassay of AVP requires preliminary steps to purify the hormone in these assay systems (Robertson et al. 1973). For these purposes, various kinds of extraction techniques, such as acetone, florisil, bentonite and resin methods, have been proposed. The liquid extraction by acetone is quite simple and practicable, but incapable of eliminating the non-specific interference to yield a constant result, unless the highly specific AVP antisera is used (Husain et al. 1973; Robertson et al. 1973). Florisil also has been used for extraction of plasma AVP, but this method requires not only a large amount of plasma (5–10 ml), but also a tedious process to readjust the pH in the final solution of the extracts (Beardwell et al. 1975; Morton et al. 1975; Baylis and Heath 1977). Bentonite was also successfully used as an absorbent for AVP (Skowsky et al. 1974), but it was said that the consistent recovery rates were not always obtained by the differences of used bentonite (Shimamoto et al. 1976; Keil and Severs 1977).
Resin column chromatography has been used frequently for the radioimmunoassay of urinary AVP (Miller and Moses 1972; Fressinaud et al. 1974; Merkelbach et al. 1975), but scarcely for the radioimmunoassay of plasma AVP except the measurement of dog plasma AVP by Shade and Share (1975). It has never been applied for the radioimmunoassay of human plasma AVP. The previous procedures of the resin column steps required so large an amount of the solution (usually, 20–25 ml of 75% acid ethanol) to elute the hormone from the column so that the resulting eluate was strongly acidic. The excessive ions in the final extract may interfere critically with the radioimmunoassay.

The present method of extraction has several advantages over the previous procedures of resin chromatography (Yoshida et al. 1963; Moran et al. 1964; Claybauch and Share 1972; Kimura et al. 1974). Elution with a minimum volume (2.0 ml) of acid acetone following the pre-elution with diluted HCl resulted in a fairly constant elution of AVP with an excellent recovery rate as shown in Fig. 2. Adjustment of pH in the final solution can be easily attained owing to its small amount.

In addition, the use of acid acetone instead of acid ethanol is convenient because the samples can be concentrated by washing with ethyl ether and dried under gentle air stream for a short time.

Recovery rates of plasma AVP were almost the same as those of previous authors (Husain et al. 1973; Morton et al. 1975; Shimamoto et al. 1976; Baylis and Heath 1977; Keil and Severs 1977), but the values for urinary AVP were much greater than the others (Miller and Moses 1972; Fressinaud et al. 1974; Merkelbach et al. 1975). The lower recovery for plasma AVP than for urinary AVP would occur in the step of TCA treatment.

The present method can be readily applicable for the determination of AVP in plasma, urine and tissues without any major modification in the procedures.

Plasma AVP levels in normal subjects at the ambulatory and dehydrated states were similar to the values reported previously. After water load, plasma AVP decreased significantly, but still detectable in almost all subjects. The values measured by the radioimmunoassay nearly coincided with those of the bioassay employing a resin extraction technique (Kimura et al. 1974), but tended to be somewhat lower than the latter.

We could not find any significant relationship between plasma AVP and its osmolality in normal subjects under physiological conditions. It seemed to be due to differences in hydration and the activity of other physiological processes of each subject which could affect AVP secretion apart from plasma osmolality (Dunn et al. 1973; Kimura et al. 1976).

In most patients with diabetes insipidus, plasma AVP could not be detected even in the dehydrated conditions, but in some cases a significant amount of plasma AVP could be proved. On the other hand, in patients with SIADH, inappropriately high plasma AVP levels were observed for the values of their
plasma osmolality. The patients with ectopic ADH producing tumor showed remarkably increased plasma AVP levels.

Daily urinary excretion of AVP in the normal and the various patients were almost equivalent to the values reported previously by several authors (Miller and Moses 1972; Fressinaud et al. 1974; Merkelbach et al. 1975). In patients with diabetes insipidus, urinary AVP could be detectable, although extremely low (about 14% of normal values), indicating that most of these patients have a residual function in the neurohypophysis to secrete a small amount of AVP into the blood despite of marked polyuria. On the other hand, significantly increased urinary AVP was found in patients with SIADH. Urinary AVP was remarkably increased in the cases of ectopic ADH producing tumor. Thus the measurement of urinary AVP as well as its plasma levels is useful to diagnose a variety of disturbances in the regulation of water and electrolytes.

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References


