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A SIMPLE AND HIGHLY SENSITIVE RADIOIMMUNOASSAY FOR 8-ARGININE VASOPRESSIN IN HUMAN PLASMA USING A REVERSED-PHASE C_{18} SILICA COLUMN

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Summary

We reported herein a simple and highly sensitive radioimmunoassay (RIA) for 8-arginine vasopressin (AVP) in human plasma and its application for clinical purpose. ODS C_{18} column was used for simple extraction of AVP from plasma. It gave high recovery rate (87.1 ± 10.4%, Mean ± SD) in the range of 1—10 pg/ml when authentic AVP was added to 0.5 ml plasma, and eliminated completely nonspecific substances which interfered with RIA. Specific antiserum was generated against AVP and permited highly sensitive RIA whose standard curve covered AVP range from 0.025 to 8 pg/ml. The within and between assay valiability was about 10% each.

Using this method, we demonstrated that plasma AVP levels in normal subjects (n=65) ranged from 0.30 to 4.20 pg/ml while those in patients with diabetes insipidus (DI) did from 0.03 to 0.21 pg/ml (n=13) or less than 0.03 pg/ml (n=3). Thus, this assay method clearly differenciated patients with DI from normal subjects. Plasma AVP levels of normal subjects (n=6) in standing, sitting and supine position after overnight fluid deprivation were 2.41 ± 1.15, 1.95 ± 0.85 and 0.97 ± 0.48 pg/ml respectively measured 30 min after each change of position. Plasma AVP concentration of normal subjects (n=6) after water load (20 ml/kg body weight) were clearly reduced from 1.89 ± 1.00 to 0.42 ± 0.21 (standing for 60 min) and also from 0.89 ± 0.41 to 0.40 ± 0.22 pg/ml (supine for 60 min).

In conclusion, we developed a simple and highly sensitive AVP assay using plasma extraction and specific RIA. This method is worthwhile for clinical application.

Key words: Plasma 8-arginine vasopression, Radioimmunoassay, Diabetes insipidus

Introduction

Determining plasma level of 8-arginine vasopressin (AVP) is very difficult because of its very low plasma level and of the presence of plasma factor which interfers RIA nonspecifically. In order to overcome these problems, many investigators tried to extract and concentrate AVP from plasma before application to assay. Acetone, florisil, bentnite and ionexchange resin extraction techniques have been reported. The recoveries of AVP in acetone extraction method are 97.6[1], 67.1[2] and 66.9%[3], and detecting sensitivities range from 0.1 to 0.5 pg/tube; In florisii[4]~[6], bentnite[7] and ionexchange resin[8], the recoveries are 46—63, 74—84 and 65.7—67.6%, respectively, and the detecting sensitivities range from 4 to 10 pg/tube, from 0.3 to 0.5 μU/ml and 0.4 pg/tube, respectively. Recently, it has been reported that reversed phase C_{18} silica column gave good recovery (87.1%) and good sensitivity (0.25 pg/tube)[9]. However, plasma of 1—2 ml or more must be used for these assay procedures.

In order to establish a routine and reliable RIA

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for AVP, we prepared an anti-AVP rabbit antiserum and studied the possible application of reversed phase C_{18} silica column in the purification of plasma AVP. Using this RIA, we report plasma AVP concentrations in normal man under several conditions, e.g. the effect of position changes and AVP concentrations after water load, hypertonic saline infusion and smoking, and those in patients with DI and syndrome of inappropriate secretion of antidiuretic hormone (SIADH).

Materials and Methods

Preparation of anti-AVP antiserum

Five mg of synthetic AVP (Ferring AB, Sweden) was conjugated with 150 mg of porcine thyroglobulin (Sigma Chem. Co., USA) by using 3 ml (100 mg/ml) of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (Peptide Inst., Japan) and the conjugate, dialysed with Visking dialization film against water for 48 hr and lyophilized for immunization antigen. Two mg/rabbit of the conjugate was resolved in 0.5 ml of saline and emulsified with an equal volume of complete Freund's adjuvant (Difco Lab., UK). Six New Zealand white rabbits were inoculated in 100 sites on the back hypodermically, and 1 mg of the conjugate was injected for each rabbit 9 times at 2—4 week intervals for 6 months. Antisera were harvested and stored at −80°C after lyophylization.

Labeled antigen

^{125}I-AVP (New England Nuclear, USA, 1820 μCi/μg, carrier free) was used.

Standard antigen and other peptides

AVP (Calbiochem-Behring, USA), 8-lysin vasopressin (LVP) (Peninsula Lab., USA), 1-deamino-8-D-arginine vasopressin (Ferring AB), oxytocin (OXT) (Peptide Inst.), 8-arginine vasotocin (AVT) (Ferring AB) and pressinoic acid (Peninsula Lab.) were used.

Reversed-phase C_{18} silica column

Silica column treated with octadecasilyl (ODS, SEP-PAK C_{18} Cartridge, Waters Associates, Inc., USA) was used.

Other chemicals

Bovine serum albumin (Sigma Chem. Co.) and PEG (# 6000, Nakarai Chem., Japan) were used. Normal rabbit serum was obtained from New Zealand white rabbits.

AVP determination in plasma

a) Extraction and purification: A mixture of 0.5 ml of plasma and 0.5 ml of 0.1 N HCl was applied to the SEP-PAK C_{18} Cartridge, previously washed twice with 12 ml of water. The column was then washed subsequently with 10 ml of 4% acetic acid to eliminate phospholipids and high molecular substances. AVP was eluted with 1.5 ml methanol; then the eluent was allowed to dry under nitrogen at 37°C. The residue was resolved in 0.3 ml of 0.1 M phosphate buffer containing 0.1% BSA.

b) RIA: An aliquot of 0.1 ml of antiserum (final dilution, 10^{-5}) was added to 0.3 ml of the reconstituted sample solution (0.1 ml of standared solution plus 0.2 ml of assay buffer in the case of standard curve). After overnight incubation at 4°C, 0.1 ml of labeled antigen (2500 cpm) was added, followed by a second overnight incubation. For B/F separation (overnight), 0.1 ml of NRS, 0.1 ml of second antibody and 0.2 ml of 25% PEG were added; bound labeled antigen was counted by a gamma counter (Fig. 1).

c) Direct RIA: Instead of 0.3 ml of 0.1 M phosphate buffer containing plasma extracts, 0.1 ml of plasma without ODS C_{18} extraction was used as a sample in the procedure b).

Subjects and plasma samples

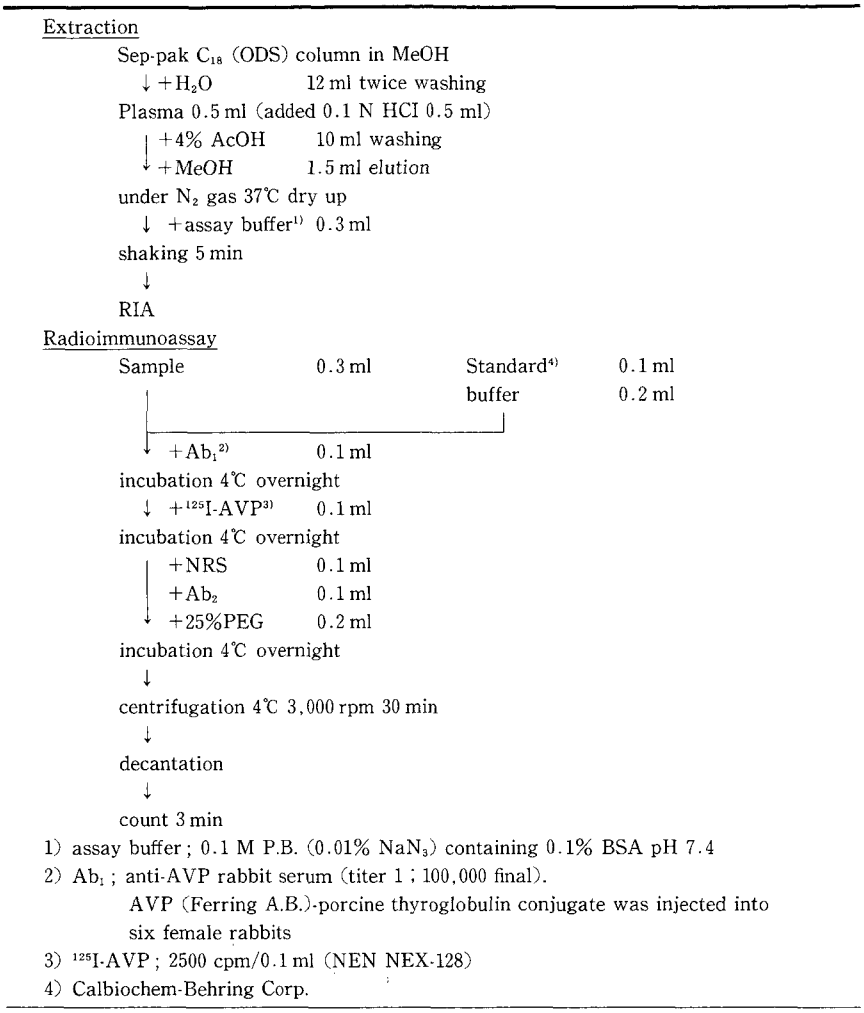
Studies were performed on volunteers ranging from 21 to 48 years and in good general health. The blood was taken from a peripheral vein into a glass tube containing 1 mg/ml blood of DETA-2K and was immediately centrifuged at 3500 g for 10 min at 4°C. The plasma was stored at −20°C until assayed. Intake of water, food and tobacco were prohibited for at least 8 hours before each sampling which began at 9 AM.

Position changes

After 60 minutes of standing, 6 males (26—40 years) were placed in sitting and then supine position for 45 min each. Blood samples were obtained from the subjects

Water load

Water (20 ml/kg body wt) was given to 3 males (26—40 years), placed in upright or supine position



1) assay buffer; 0.1 M P.B. (0.01% NaN_3) containing 0.1% BSA pH 7.4
2) Ab_1; anti-AVP rabbit serum (titer 1 ; 100,000 final).
AVP (Ferring A.B.)-porcine thyroglobulin conjugate was injected into six female rabbits
3) ^{125}I-AVP; 2500 cpm/0.1 ml (NEN NEX-128)
4) Calbiochem-Behring Corp.

Fig. 1 Assay procedure for measurement of plasma AVP.

for 30 min, and blood was drawn every 30 min for up to 90 min.

Hypertonic (2.5%) saline infusion

After 30 min in the supine position, infusion of 2.5% saline was started in the antecubital vein at a rate of 0.2 ml/min/kg body wt for 45 min in 6 males (26—35 years). The blood samples were obtained 30 min after the end of infusion.

Nicotine load

The subjects (26—31 year males, n=3) smoked two cigarattes within 5 min.

Clinical study

Plasma samples were obtained from patients with diabetes insipidus (n=16) and SIADH (n=2).

Plasma osmolalities

Plasma osmolalities were measured by freezing point depression using an Advanced Osmometer, Model 3W (USA).

Results

Specificity of antiserum

The cross-reactivities of various analogues of AVP with the antiserum are shown in Fig. 2. LVP was as potent as AVP in inhibiting ^{125}I-AVP binding, but the potencies of other analogues were 1% or less, judged by their ability to achieve half maximal (B/B_o=50%) inhibition.

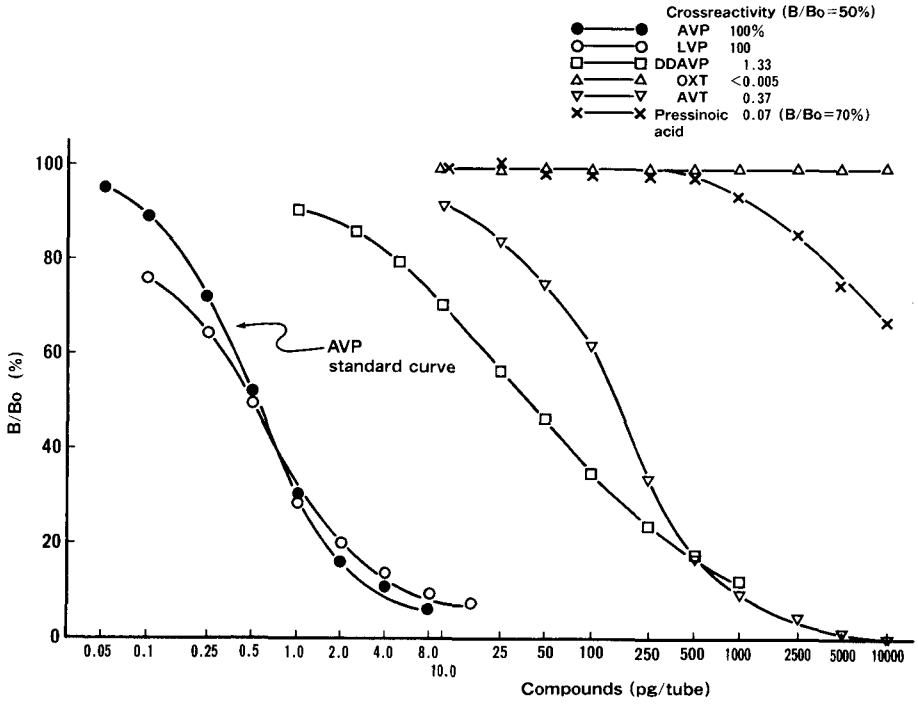


Fig. 2 Specificity of anti-AVP antiserum.

Measurement of AVP

Addition of known amounts of ^{125}I-AVP (about 10000 dpm) to plasma resulted in a recovery of 95.1 ± 1.7% (n=5) after extraction with SEP-PAK C_{18} Cartridge.

Unextracted plasma (0.5 ml) or ODS C_{18}-column-extracted plasma was applied to a Sephadex G-25 column, and the eluate was assayed for AVP-immunoreactivity. As shown in Fig. 3, immunoreactive substances in the unextracted plasma which was eluted in fraction 6—11 (void volume) were completely eliminated in the ODS C_{18}-column-extracted plasma.

When unextracted plasma was added directly in the present assay system, four of 24 samples of normal adults gave AVP values higher than 20 pg/ml and two of the four samples showed the value higher than 80 pg/ml. In contrast, the values ranged from 0.76 to 4.69 pg/ml when determined using plasma extracts. The average values of plasma AVP were 4.50 pg/ml in unextracted plasma and 1.06 pg/ml in extracted plasma, the difference being 3.43 pg/ml (Table 1). The value agrees well with the concentration of AVP-like immunoreactive material (3.24 pg/ml) which was eluted in the void volume fractions on Sephadex G-25 column (Fig. 3 upper panell).

Recovery in the entire procedure was also studied by adding known amounts of unlabeled AVP to plasma; the average recovery was 87.1% when 1.0—10.0 pg/ml of AVP was added to plasma containing 0.47 pg/ml (Table 2).

Fig. 4 shows that the delayed addition of ^{125}I-AVP (disequilibrium assay; one day incubation with standards prior to the addition of ^{125}I-AVP) significantly improves the assay sensitivity, as compared to the simultaneous addition of tracer and standards (equilibrium assay: one day incubation). The amount of AVP required to achieve half maximal ^{125}I-AVP binding inhibition was as low as 0.62 pg/tube in the present disequilibrium assay, while the value was 3.5 pg/tube in ordinary equilibrium assay. Thus, the sensitivity was improved by more than five-fold. The period of either first (1—3 days) or second (1—3 days) phase incubation had little effect on the assay.

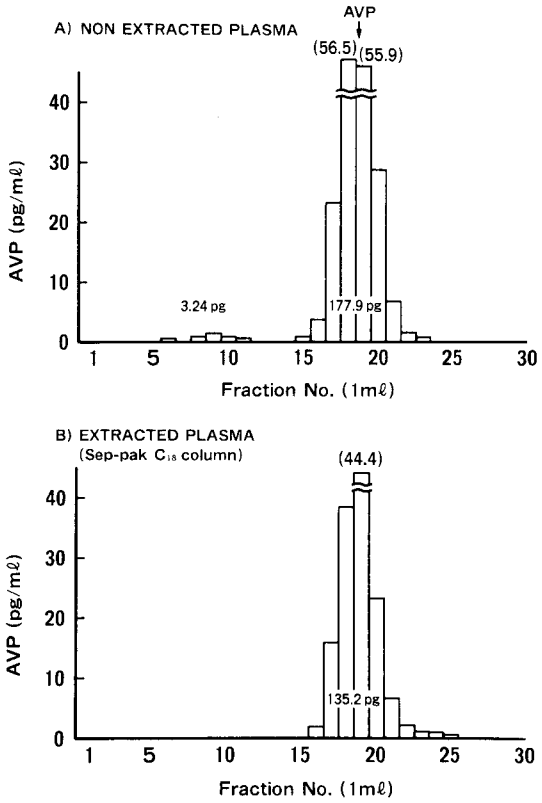


Fig. 3 Immunoreactivity after gel chromatography of plasma. The vertical arrow across the top represents standard AVP (RIA). Column: Sephadex G-25, 1 × 80 cm, Elution buffer: 0.1 M phosphate beffer, pH 7.4, Fraction volume: 1 ml.

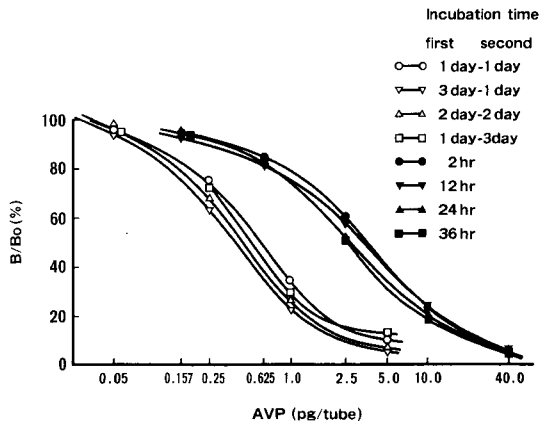


Fig. 4 Effects of incubation time on sensitivity.

Table 1 Comparison of plasma AVP with ODS C_{18} extraction and direct RIA

	AVP concentration(pg/ml)		⊿AVP(pg/ml)
	direct	ODS C_{18}	
	4.42	1.64	2.78
	4.03	1.16	2.87
	4.92	0.49	4.43
	*80↑	4.49	—
	5.68	0.52	5.16
	6.77	0.74	6.03
	*20.13	3.97	16.15
	4.09	1.13	2.96
	*80↑	4.65	—
	6.06	4.26	1.80
	3.86	0.38	3.48
	2.83	0.79	2.04
	3.73	0.44	3.29
	4.61	1.03	3.48
	5.53	0.63	4.90
	4.52	1.74	2.78
	3.97	2.43	1.54
	*27.30	0.76	26.54
	3.20	0.84	2.36
	3.68	0.16	3.52
	3.92	0.27	3.65
	5.13	1.40	3.73
	4.49	0.68	3.81
	4.46	0.50	3.96
Mean±S.D.	4.50±0.95	1.06±0.92	3.43±1.10

*these data are not included.

Table 2 Recovery of AVP added to a known plasma

Amount of added AVP (pg/ml)	No.	Estimated values (pg/ml)	Recovery (%)
0	1	0.52	(−)
	2	0.44	
	3	0.44	
1.0	1	1.31	84.0
	2	1.21	74.0
	3	1.48	101.0
2.5	1	2.58	84.4
	2	2.50	81.2
	3	2.60	85.2
5.0	1	5.23	95.2
	2	5.18	94.2
	3	5.55	101.6
10.0	1	8.82	83.5
	2	9.18	87.1
	3	7.90	74.3
		Mean±S.D.	87.1±8.8

The minimum determinable dose of the calibration curve was 0.05 pg/tube as judged by B/B_0=95% intercept. The measuring range of the present method was from 0.05 to 8.0 pg/tube (Fig. 5).

Fig. 5 depicts that the dose response curves of two plasma extracts serially diluted with 0.1 M phosphate buffer (pH 7.4, 0.1% BSA) were parallel to those for standard AVP.

Three pooled plasma containing 0.48—15.5 pg/ml were simultaneously measured at eight points. The wihtin and between assay variabilities ranged from 7.85 to 14.4 and 8.42 to 13.8%, respectively (Table 3).

No significant loss of AVP immuno-reactivity of the different plasam (5.2 and 1.1 pg/ml) was found under −30°C after one month storage, but significant loss was found when stored at 4°C and room temperature; in other words, we found a 6—8% loss at 4°C after 2—3 weeks and a 0—16% loss at room temperature after 3 days. After one month, 8—32 and 33-45% losses were observed at 4°C and room temperature, respectively.

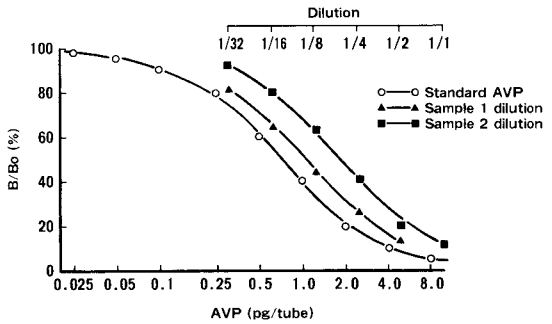


Fig. 5 Dose response curve and dilution test with assay buffer solution.

Table 3 Reproducibility of within and between assay

	Within-assay		
n	8	8	8
Mean values(pg/ml)	0.48	4.64	15.5
S.D.	0.07	0.36	1.73
C.V.(%)	14.4	7.85	11.2

	Between-assay		
n	5	6	5
Mean values(pg/ml)	0.53	3.81	6.18
S.D.	0.07	0.35	0.51
C.V.(%)	13.8	9.31	8.24

Clinical studies

The normal values of plasma AVP (21—48 years, 34 males and 31 females) ranged between 0.30 and 4.20 pg/ml showing a logarithmic distribution with the mean value of 1.25 pg/ml (Fig. 10). There was no significant difference between male and female values (data not shown).

The plasma AVP values after 60 min upright position was 2.41 ± 1.15 pg/ml which declined to 1.95 ± 0.85 after 30 min in the sitting position and declined further to 0.97 ± 0.48 pg/ml after 30 min in the supine position. A significant fall was observed from sitting to supine position (Fig. 6).

The plasma AVP concentrations after the water load fell from 1.89 ± 1.00 pg/ml (upright) and 0.89 ± 0.41 (supine) to 0.42 ± 0.21 and 0.40 ± 0.22, respectively, after 60 min. After 90 min, slightly rising trends, 0.44 ± 0.24 and 0.77 ± 0.36 pg/ml, were observed in both positions. The plasma AVP in the upright position varied much more markedly than in the supine position. While plasma osmolalities showed approximately parallel changes with plasma AVP concentrations, urine osmolalities remarkably fell from 855 ± 127 to 161 ± 34 mOsm/kg in the upright position, and from 1005 ± 33 to 243 ± 33 mOsm/kg in the supine position (Fig.

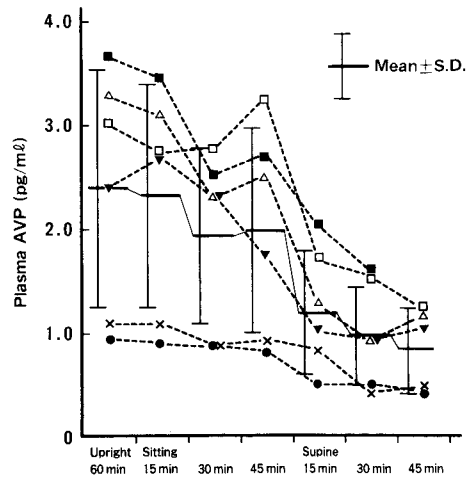


Fig. 6 Variability of plasma AVP in the upright, sitting and supine position.

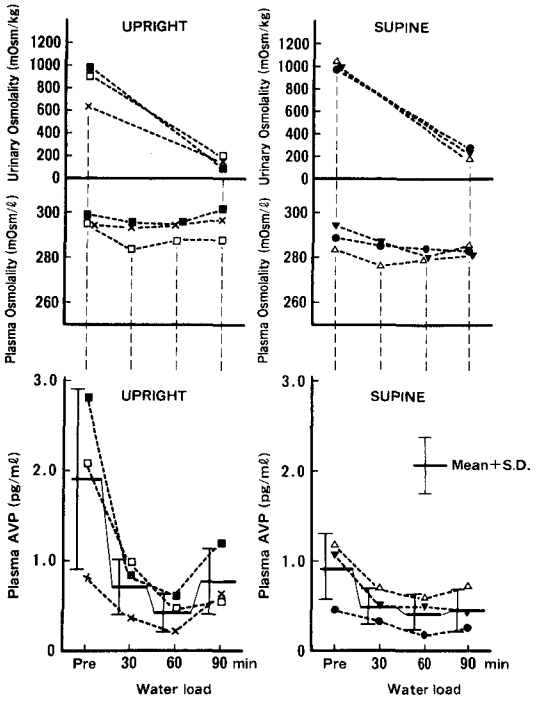


Fig. 7 Plasma AVP in normal subjects after water load (20 ml/kg body wt).

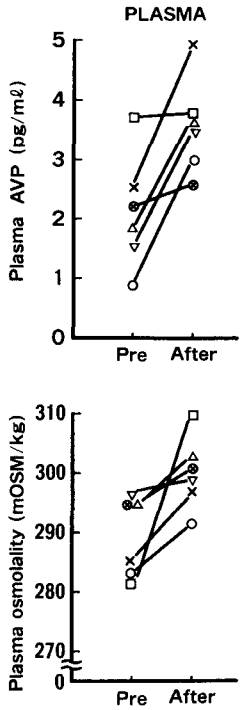


Fig. 8 Hypertonic saline infusion test.

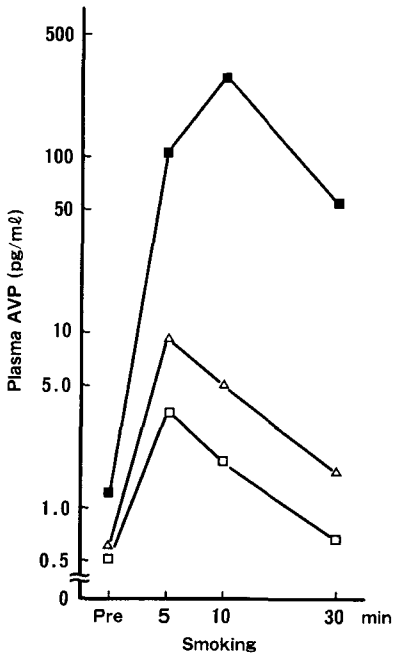


Fig. 9 Plasma AVP in normal subjects after smoking (two cigarettes).

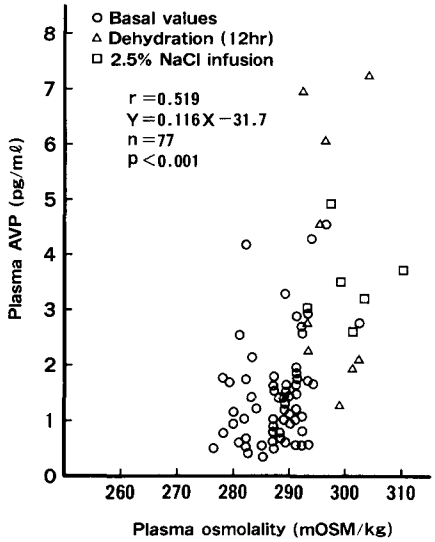


Fig. 10 Relationship between plasma APV and plasma osmolality.

7).

The basal plasma AVP values rose from 2.17 ± 0.08 pg/ml to 3.58 ± 0.73 pg/ml after 2.5% saline infusion; the plasma osmolalities also rose from 290 ± 6 to 301 ± 5 mOsm/kg (Fig. 8).

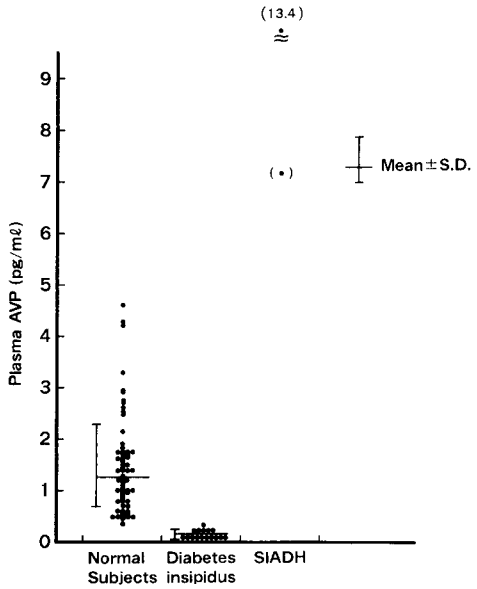


Fig. 11 Plasma AVP in normal subjects, patients with diabetes insipidus and patients with inappropriate ADH secretion.

Smoking increased plasma AVP extremely (6—200 fold increase) with peaking at 5 to 10 minutes after smoking. The light (less than 10 cigarettes a day) smoker's plamsa AVP showed the highest rise (Fig. 9).

A relationship between plasma AVP levels and their osmolalities was found to be Y=0.116X − 31.7, r=0.519 (normal adults, n=77) (Fig. 10).

All plasma AVP values of the 16 patients with DI showed less than 0.31 pg/ml using 0.5 ml of plasma. However, when using 2 ml of plasma, AVP levels became determinable. One sample showed 0.31 pg/ml; 12 ranged from 0.03 to 0.21 pg/ml, and three others were less than 0.03 pg/ml. Samples of 2 patients with SIADH revealed 7.1 and 13.4 pg/ml (Fig. 11).

Discussion

A simple and highly sensitive RIA for the measurement of plasma AVP was established by using highly sensitive anti-AVP antiserum and an ODS C_{18} column for extraction and purification of plasma AVP.

It was necessary to eliminate interfering substances from plasma for AVP measurement (see Introduction). Immunoreactive substances in the unextracted plasma which was eluted in fraction 6—11 (void volume), were completely eliminated when plasma was through ODS C_{18} column. Robertson's results[1] also showed that the immunoreactive substances, eliminated with acetone-ether extraction, were eluted in the void volume. This immunoreactive and interfering substances was calculated by AVP-RIA to be 3.24 pg/ml (Fig. 3) or 3.43 pg/ml (Table 1). This value was compatible with that which Robertson et al[1] reported (3.1 pg/ml). Thus the immunoreactive substances eliminated by ODS C_{18} Cartridge seem to be similar to the substances extracted by acetone-ether. These observations suggest that the discrepancy between the AVP values in unextracted plasma and in plasma extracts may be due to the presence of nonspecific interfering substances.

We have nerve had any problems with the quality of the cartridge in our laboratory routine tests through more than 15,000 samples in the past two years. An adsorption capacity of the cartridge for AVP was not studies, but no difference was found in the determined plasma concentrations of AVP per ml whether 0.5 ml or 2 ml sample was extracted with the column. Glänzer et al[9] reported an average recovery of 87.1% using 2 ml of plasma and ODS C_{18} Cartridge; they also reported normal values of 1.2 ± 0.6 (male) and 1.7 ± 0.7 (female) pg/ml, and a sensitivity of 0.3 pg/ml. Thus, it can be concluded that the extraction method using the ODS C_{18} Cartridge is simple, stable and excellent for eliminating nonspecific interfering materials.

RIA with the antiserum we have produced was highly sensitive. It showed a 100% cross reaction with LVP, whereas it cross-reacted little with pressinoic acid which has the same ring structure as AVP, AVT which is substituted by isoleucine for 3-phenyl alanine of AVP, or OXT substituted by isoleucine for 3-phenylalanine of LVP. Therefore, this suggests that the antiserum does not specifically bind only to the ring portion or the sidechain selectively but instead binds specifically to the overall structure of AVP or LVP.

We can thus assume that our antiserum has specificity different from that of Glick[1][2] having cross

reactivities of 1/3, 1/6, and 1/100 to LVP, AVT and OXT, respectively, and also from that of Kimura[8] having no cross reactivity of LVP.

The plasma AVP values of 65 normal adults were fitted to six kinds of distribution curves (normal, logarithmic, parabolic, X^3, $X^{1/2}$ and $X^{1/3}$); the best fit was found to be logarithmic resulting in a normal range of 0.30—4.20 pg/ml (Mean ± 2SD). Our results also demonstrated that plasma AVP levels change significantly by change of position and of hydration, and smoking. When blood sampling is performed to determine plasma AVP concentrations, these influences should be considered.

The present method permits the determination of the plasma AVP levels of patients with DI which had been below the sensitivities of conventional methods. It allows easily to discriminate between the normal person and the patient without any troublesome test such as water-deprivation test.

Finally, we studied the relationship between the concentrations of plasma AVP extracted with acetone-ether by our antiserum and Glick's antiserum. There was a good correlation between the values obtained by the two antisera; $Y=0.976X - 0.204$, $r=0.997$ (Y: the value by our antiserum, X: the value by Glick's antiserum) in the range of 0.3 – 12.6 pg/ml[10]. This shows that the present antiserum can also be applied to the acetone-ether extraction method.

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逆相 C_{18} シリカカラムを用いる簡易，高感度 Radioimmunoassay による ヒト血漿中8-arginine vasopressin の測定法

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我々は，本報において，ヒト血漿中8-arginine vasopressin（AVP）の簡易かつ高感度 radioimmunoassay（RIA）およびその臨床的応用について報告した．血漿からの AVP の簡易抽出法として，ODS C_{18} カラムを用いた．この方法で，authentic AVP 1～10pg/ml を0.5ml の血漿に加えたときの回収率は87.1±10.4%（Mean±SD）であり，また RIA を妨害する非特異物質を完全に除去した．AVP に対する特異抗血清を作成し，測定範囲が0.025～8pg/ml の高感度 RIA を確立した．アッセイ内およびアッセイ間変動系数はともに約10%であった．

この方法を用いて，我々は健常人血漿中 AVP 値は0.30～4.20pg/ml（n＝65）であり，尿崩症患者（n＝16）では0.03～0.21pg/ml（n＝13）あるいは0.03pg/ml（n＝3）以下であることを示した．このように，本法は尿崩症患者と健常者とを明確に区別した．一夜飲水制限後の健常人（n＝6）の立位，坐位および臥位各30分維持後の血漿中 AVP 値はそれぞれ，2.41±1.15, 1.95±0.85および0.97±0.48pg/ml であった．飲水（20ml/kg body weight）前後の健常人（n＝6）血漿中 AVP 値は，1.89±1.00から0.42±0.21（立位，60分後）へ，また0.89±0.41から0.40±0.22（臥位，60分後）へと明らかに減少した．結論：我々は血漿抽出と特異的 RIA を用いて，簡易かつ高感度な AVP 測定法を確立した．本法は臨床的応用に有用である．