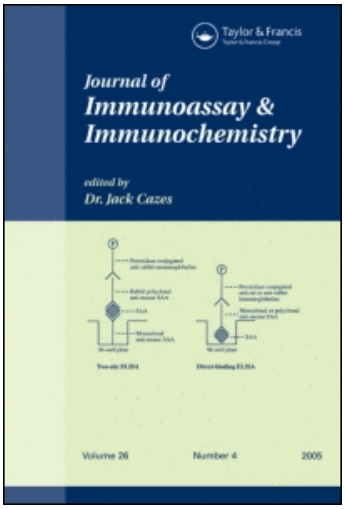


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# A New Sensitive Radioimmunoassay for Plasma Arginine Vasopressin

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# A NEW SENSITIVE RADIOIMMUNOASSAY FOR PLASMA ARGININE VASOPRESSIN

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## ABSTRACT

A new extremely sensitive radioimmunoassay to measure plasma AVP has been developed. Antiserum to AVP of high affinity (Keq = 1.1 x $10^{12}$ l/mol), raised in rabbits, showed little cross-reaction with related analogues (LVP < 0.03%, AVT, DDAVP, OXT < 0.01%). The specific activity of $^{125}$I-AVP (chloramine T method) was 1400 - 1750 Ci/mmol. The limit of detection of plasma AVP, after extraction of 2 ml plasma with Florisil, was 0.3 pmol/l. Coefficient of variations of plasma control (2 pmol/l) were 9.7% (intra-assay) and 15.3% (inter-assay), n = 15.

Osmotic stimulation by hypertonic saline infusion caused a linear response in plasma AVP in normal subjects, but plasma AVP remained undetectable in patients with cranial diabetes insipidus. Suppression of AVP secretion by a standard oral water load and by alcohol and fluid in volunteers produced low or undetectable values of plasma AVP. In a patient with the syndrome of inappropriate antidiuresis, plasma AVP concentration was grossly elevated.

## INTRODUCTION

The antidiuretic hormone of most mammals including man is arginine vasopressin (AVP). Its concentration in human plasma appears to be extremely low, of the order of 1 - 10 pmol/l. Particularly sensitive assay methods are required to detect these concentrations. Antidiuretic bioassays using the water-loaded,

ethanol-anaesthetised rat are capable of detecting plasma AVP concentration but the methods are capricious, and the assay of large numbers of samples difficult and time-consuming (1). Radioimmunoassays for this peptide were first established approximately 10 years ago but only a few have been capable of detecting physiological levels of AVP on small volumes of plasma (2, 3, 4, 5, 6). All assays, except that described by Fyhrquist, Wallenius & Hollemans (7), have required extraction of the peptide from plasma to remove substances that appear to interfere with the immunoassay. Despite the necessity to extract, immunoassays provide the most convenient, quick, reliable and sensitive method to measure AVP although newer methods are being developed which may be more sensitive, e.g. cytochemical bioassay described by Baylis, Pitchfork, Chayen & Bitensky (8) or more accurate in defining precisely the molecular species, e.g. high performance liquid chromatography described by Swann, Pickering, Robinson & Corran (9).



This paper describes the characteristics of a new high affinity antiserum to AVP, which readily detects low values of AVP in small samples of plasma. Osmoregulatory studies provide physiological validation of the method.

## MATERIALS AND METHODS

Synthetic AVP (rat pressor potency 400 u/mg), donated by Dr. H. Vilhardt (Ferring AB, Sweden) was used to raise antisera and to prepare standards and iodinated AVP. Vasopressin analogues 1-

deamino-D-arginine vasopressin (DDAVP), lysine vasopressin (LVP) and its triglycyl derivative ($Gly_3$ - LVP), pressinoic acid (PA), the tripeptide Pro-Arg-Gly (PAG) and oxytocin (OXT) were a gift from Dr. Vilhardt. Arginine vasopressin (AVT) was provided by Dr. MacIntyre (St. George's Hospital, London). The purity of synthetic AVP was checked by chromatography on Sephadex G-25 and DEAE-Sephadex A-25 (Pharmacia Fine Chemicals) and by radioelectrophoresis run in 70 mmol/l barbitone buffer, pH 8.6, on Whatman 3MC paper. AVP was stored in 0.2 mol/l acetic acid containing 2 g/l bovine serum albumin (BSA, Fraction V, Sigma Chemical Co.) at -70°C in stock concentrations of 0.5 mmol/l. All other reagents were Analar grade.

Iodination of AVP was performed by a modification of the Chloramine T method (10). Iodinated AVP was readily separated from radioactive iodine and AVP by chromatography on Sephadex G-25 and eluted with 40 mmol/l acetic acid containing 1 g/l BSA (11). It was stored at -30°C in assay buffer. Specific activity ranged from 1400 to 1750 Ci/mmol.

Antisera was raised in 12 female New Zealand white rabbits. The antigen was prepared by conjugation of AVP to bovine thyroglobulin (Sigma Chemical Co.) using the carbodiimide reaction (12) in the molar ratio, AVP : bovine thyroglobulin : carbodiimide = 60 : 1 : 200. Incorporation of AVP, monitored by $^{125}I$-AVP, was 25%. The reaction mixture was purified on Sephadex G-25. AVP conjugate containing 20 nmol AVP, was mixed with Freund's adjuvent before injection by the multiple site intradermal route at

intervals of 4 months. All rabbits became polyuric. After 1 year, the rabbit which had been most polyuric, passing 1.5 - 2.0 litres of urine/24 h, produced an exceptionally good antiserum, which was stored neat or diluted in assay buffer at -70°C.

The radioimmunoassay buffer was 50 mmol/l Tris-HCl containing 3.5 g/l BSA and 0.3 mmol/l sodium azide, pH 7.5. The assay was performed in Luckham LP-3 polystyrene tubes; and set up as follows. Four zero standard (initial binding) tubes contained 200 µl buffer and 100 µl AVP antiserum (final dilution 1:500,000). Triplicate tubes for each AVP standard contained the above reagents except that the 200 µl buffer was replaced by AVP (0.03 - 8 fmol) in 200 µl of buffer. Plasma extracts of 200 µl replaced the standards. Non-specific binding tubes had 300 µl buffer only. After pre-incubation for 24 h at 4°C, 100 µl $^{125}$I-AVP (1000 - 2000 cpm, equivalent to 0.5 - 1 fmol AVP) was added to each tube. Incubation continued for a further 18 h at 4°C after which the bound AVP was separated from the free hormone by adding 100 µl goat anti-rabbit gamma-globulin (Dr. R.A. Bradwell, University of Birmingham) diluted 1:40 with assay buffer containing 100 mmol/l EDTA and 0.2% normal rabbit serum, pH 7.5. After incubation for 48 h at 4°C the precipitate was separated by centrifugation at 2000 rpm at 4°C. The supernatant was removed by aspiration and the tubes were counted in a gamma counter (Nuclear, Chicago). The ratio of antiserum specifically bound $^{125}$I-AVP to total $^{125}$I-AVP was calculated for each AVP standard, expressed as a percentage of initial binding, and plotted against the appropriate AVP standard.

AVP controls of 1.25 fmol/400 µl assay tube were measured in duplicate in each assay.

Venous blood for extraction and assay was prepared by transferring it immediately into chilled heparinised plastic tubes which were centrifuged at 2000 rpm for 10 min at 4°C. The plasma was separated and stored in plain plastic tubes at -30°C for extraction within 1 month. Immunoreactive AVP in plasma stored under these conditions did not deteriorate for 3 months. AVP was extracted from plasma by adsorption on to Florisil 100 - 200 mesh (Sigma Chemical Co.) which had been thoroughly washed with distilled water, HCl and NaOH, and heat activated at 120°C for 18 h. The extraction method previously described by Beardwell (13), was modified as follows. Plasma (1 - 2 ml) was mixed with 10 - 20 mg Florisil for 10 min, and the supernatant removed. The Florisil was washed twice with distilled water and acidified with 0.2 mol/l HCl. AVP was eluted with 1ml 90% aqueous-acetone twice. Diethyl ether (2 ml) was added to the pooled eluate, vigorously mixed and the solution allowed to settle for 5 min. The upper phase was removed by aspiration and the lower phase dried completely at 40°C under a stream of $N_2$. The residue was reconstituted with 800 µl of assay buffer. A plasma control (2 pmol/l) was measured in each assay in duplicate.

Preliminary studies assessed the recovery of unlabelled AVP added to plasma in the range 2 - 100 pmol/l. Physiological validation was obtained by observing the response of plasma AVP to increasing plasma tonicity in 2 normal subjects (N), 3 patients with severe cranial diabetes insipidus (CDI) who all had polyuria

in excess of 12 litres/24 h and one patient with hyponatraemia, proven oat cell carcinoma of the bronchus and who fulfilled the criteria of the syndrome of inappropriate antidiuresis (SIAD) described by Bartter & Schwartz (14). After overnight fasting with only water to drink, each subject emptied the bladder, rested supine, and 2 cannulae were inserted into veins in each arm. Hypertonic saline (0.06 ml/kg/min) was infused for 2 h into one arm, whole 10 ml venous blood samples were withdrawn at 30 min intervals during the study. The samples were processed as described above and a further 2 ml plasma aliquot was saved to measure osmolality (Research Osmometer 3R, Advanced Instruments). In another series of studies 6 normal subjects were given alcohol and fluid. Four pints of beer were drunk over 2 h. Venous blood was drawn before ingestion, at the end of drinking, and after a further 10 hours during which the subjects slept. Three other subjects received a standard oral water load (20 ml/kg) over a 15 min period. Blood was taken before and 1 h after the load, when the diuresis was maximal. All patients and volunteers gave informed consent for studies approved by the Local Ethical Committee. Results are expressed as mean $\pm$ 1 standard deviation, unless stated otherwise.



## RESULTS

All rabbits produced antisera to AVP. One rabbit developed profound polyuria and had raised a particularly sensitive, specific antiserum of high titre. Under equilibrium assay conditions,

Scatchard analysis gave an equilibrium constant of $1.1 \times 10^{12}$ l/mol; the correlation coefficient of the regression line relating the ratio of bound AVP to free hormone to the total AVP bound to the antiserum was 0.92, $p < 0.01$ (15). Figure 1 shows the specificity of the antiserum for AVP. Cross-reactivity with LVP was $< 0.03\%$ and with all other analogues was $< 0.01\%$. There was minimal binding with the tripeptide tail of the AVP molecule (PAG) but no binding with the ring (PA). Binding studies were also performed with growth hormone, adrenocorticotrophin, prolactin, placental lactogen, glucagon, thyroxine, gonadotrophins, cortisol and catecholamines, but no cross-reactivity was observed.

The standard curve is given in Figure 2. Specific initial binding was $19.9 \pm 1.7\%$, and non-specific binding was 1.2% or less. The limit of detection of the assay has been defined arbitarily as depression from initial binding by 20%. Thus, this assay could readily detect 0.1 fmol AVP/tube (assay volume 400 µl). Displacement of 50% of the bound $^{125}$I-AVP occurred at $0.71 \pm 0.02$ fmol/tube (n = 15). The intra-assay coefficient of variation of the AVP control (1.25 fmol/tube) was 8.3%, n = 20, and the inter-assay coefficient of variation was 12.4%, n = 15. Extracted plasma samples gave an intra-assay coefficient of variation of 9.7%, n = 10, and inter-assay coefficient of variation of 15.3%, n = 15.

Extraction of water blanks, heparin, and solutions of BSA (10 - 100 g/l) did not depress binding in the immunoassay. The pH of all extracts was neutral. Table I gives the results of the

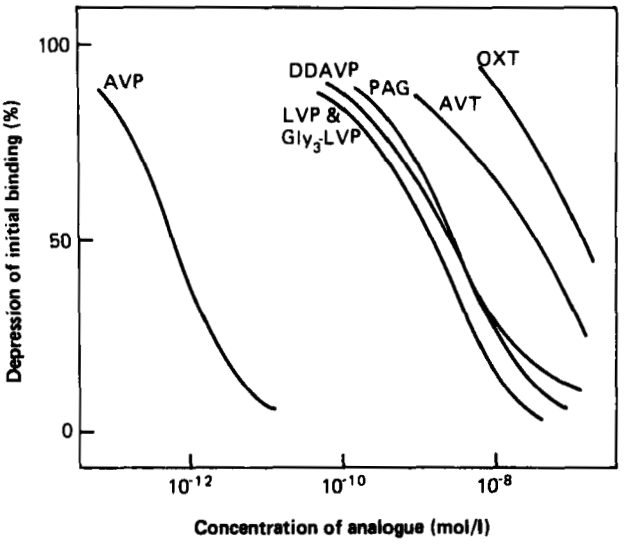


Figure 1

Binding studies of AVP and related analogues to new antiserum.

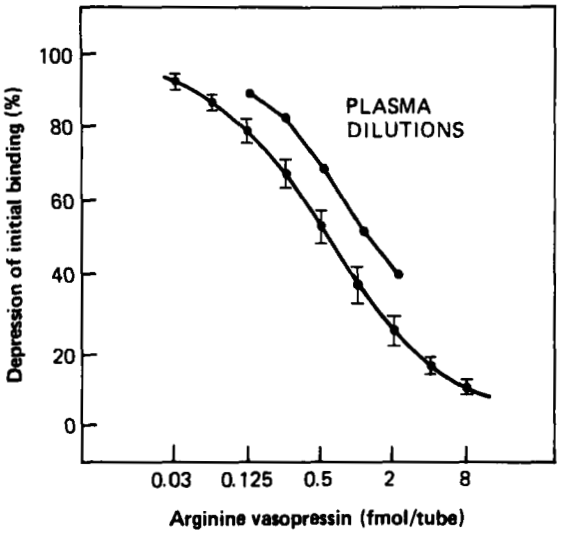


Figure 2

Standard curve using new antiserum. Points are represented as mean ± 1 SD, n = 15. Total volume per tube was 400 µl. Specific binding was 19.9 ± 1.7%, non-specific binding 1.1%, inter-assay coefficient of variation 12.4%. Dilutions of plasma extracted from a patient with the syndrome of inappropriate antidiuresis gave values which were parallel to the standard curve.

recovery of unlabelled AVP to 2 ml plasma aliquots over the range 2 - 100 pmol/l. At normal physiological concentrations (2 - 4 pmol/l) there was no loss of AVP in the extraction, but at concentrations of 10 pmol/l recovery was 86% and at 100 pmol/l it fell to 72%. Progressive dilution of extracts ran parallel to the standard curve. It was decided to calculate results of plasma AVP concentrations without allowing for those minor changes in recovery. There was no loss in immunoreactive AVP in plasma samples or residues of extracted plasma samples stored at $-30^{\circ}$ up to 3 months.



Infusion of hypertonic saline into 2 normal subjects caused a progressive rise in osmolality and a linear increase in plasma AVP concentration (B and C, Figure 3). The regression functions of these two individuals were pAVP = 0.27 (pOS - 283), r = + 0.99, $p < 0.001$ and pAVP = 0.26 (pOS - 281), r = 0.97, $p < 0.01$, respectively (pAVP represents plasma AVP and pOS, plasma osmolality). The 3 patients with severe CDI all had undetectable plasma AVP despite marked hypertonicity, except patient D whose plasma AVP rose to 0.4 pmol/l at the end of the infusion (Figure 3). Patient A who had SIAD, had grossly elevated values of plasma AVP which did not correlate with plasma osmolality. Chromatography of this extracted plasma on Sephadex G-25 eluted in the position of synthetic AVP.

The ingestion of beer in 6 normal subjects caused a substantial diuresis. Free water clearance rose from a basal of - 0.99 $\pm$ 0.12 ml/min to + 10.1 $\pm$ 1.5 ml/min 3 h after starting to drink.

TABLE 1

Recovery of unlabelled AVP added to 2 ml aliquots of fresh plasma

| Added AVP (pg/ml) | Percentage Recovery (mean± SD) | n |
|---|---|---|
| 2 | 100.2 ± 2.3 | 8 |
| 4 | 98.4 ± 4.1 | 8 |
| 10 | 86.5 ± 4.5 | 8 |
| 20 | 74.8 ± 5.4 | 8 |
| 100 | 72.5 ± 6.1 | 8 |



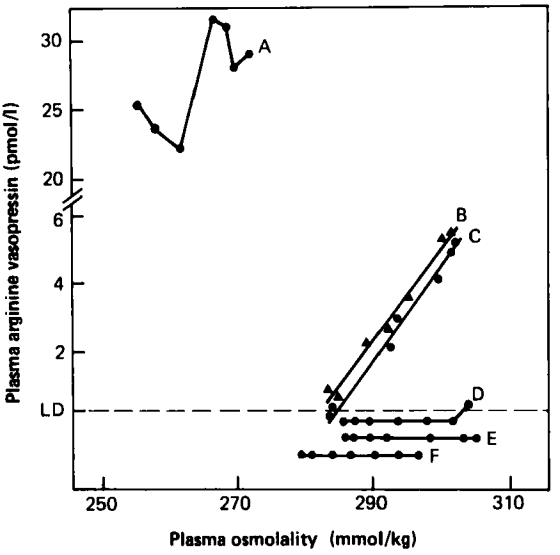


Figure 3

The relation of plasma osmolality to plasma AVP after infusion of hypertonic saline (5%) into a patient with the syndrome of inappropriate antidiuresis (A), 2 normal volunteers (B & C), and 3 patients with severe cranial diabetes insipidus (D, E, & F).

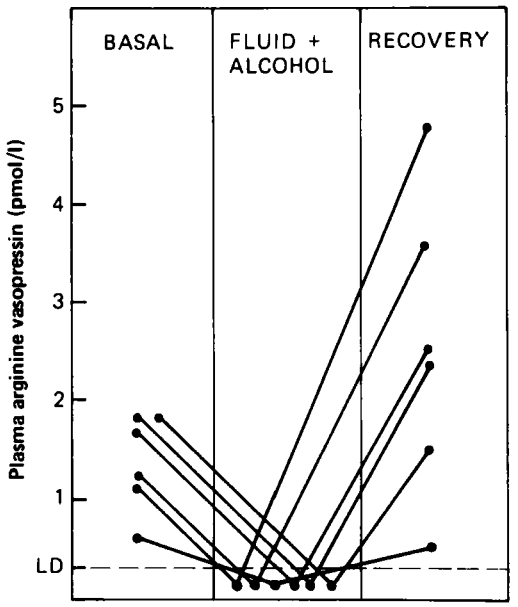


Figure 4

Plasma AVP response to ingestion of 4 pints of beer in 6 normal volunteers, before ingestion (basal), after drinking the beer within 2 h (fluid and alcohol) and following a further 10 h rest supine (recovery).



This returned to - 1.00 ± 0.03 ml/min after a further 10 h. The changes in plasma AVP concentrations are given in Figure 4. All subjects had undetectable plasma AVP after drinking beer; their values returned to normal after 10 h.

The 3 subjects who had an oral water load, showed a decrease in plasma AVP from a basal value of 2.5 ± 0.7 to 0.5 ± 0.1 pmol/l, accompanied by changes in plasma osmolality from 288 ± 1.5 to 280 ± 0.4 mmol/kg.

## DISCUSSION

This new radioimmunoassay for plasma AVP is extremely sensitive and allows low physiological concentrations of the hormone to be measured. The improvement in the assay is due principally to the antiserum which appears to have very little cross-reactivity with closely related analogues and which has a high affinity. Most antisera to AVP bind to LVP quite readily. Thomas & Lee (5) showed that their better AVP antiserum cross-reacted 20% with LVP, which is in keeping with other workers who have described their antisera in detail (3, 16, 17). This new antiserum binds LVP less than 0.03% and appears to be highly specific for AVP. From the cross-reactivity data available there may be preferential binding to the tripeptide tail portion of the molecule because there is greater binding to the Pro-Arg-Gly tripeptide than to pressinoic acid, the molecular ring of AVP. Work on structure-function relationships with AVP has emphasised the importance of the tripeptide tail, particularly the basic amino-acid at position 8 of the molecule, in determining diuretic potency (18). Therefore, it would appear that this antiserum should be very suitable for measuring changes in the antidiuretic hormone.

The equilibrium constant of this antiserum is extremely high, which is essential for a sensitive immunoassay. The specificity and sensitivity of the antiserum were reflected in the gross diabetes insipidus that developed in the rabbit which produced the antiserum (19). The reason for one rabbit producing such a good antiserum is unclear and is probably fortuitous. However, the

authors did modify their antisera production technique in comparison to their previously described method (11). The AVP-thyroglobulin conjugate was purified by chromatography before immunisation, very small doses of immunogen were used, and immunisation occurred at intervals of 4 months.

The iodination technique used in this assay is identical to that previously described (11) and continued to give labelled AVP of high specific activity close to the theoretical value of monoiodinated AVP (1800 Ci/mmol).



The standard curve of the immunoassay is reproducible and the coefficient of variation of the inter- and intra-assay controls are satisfactory. The limit of detection is 0.1 fmol/assay tube, which is extremely low. It is therefore now possible to use small quantities of plasma to detect physiological changes in AVP concentration. With 2 ml aliquots of plasma and the high recovery of unlabelled AVP at around phyisological plasma concentrations of AVP, the plasma detection limit is of the order of 0.3 pmol/l. The dilution of extracted plasma samples run parallel to the standard curve and the inter- and intra-assay coefficient of variations of extracted plasma aliquots are good (15.3% and 9.7%, respectively).

Validation of the assay has been obtained by observing changes in plasma AVP in response to osmotic change. In normal individuals there was a linear increase of plasma AVP concentration to slow hypertonic saline infusion. The relationship of plasma AVP to plasma osmolality may be described by the function of pAVP = m (pOS - b) where m represents the slope and b the abscissal

intercept (20). The values for m (0.26, 0.27) and b (281, 283) are very similar to those quoted by Robertson et al (20) and are in keeping with recognised physiological values (21).

A stringent test of the quality of an AVP radioimmunoassay is its ability to record low or undetectable plasma AVP values following suppression of AVP release because many radioimmunoassays appear to suffer from non-specific interference at low physiological levels (10). An oral water loaded suppressed plasma AVP in 3 normal subjects from mean values of 2.5 to 0.5 pmol/l. Furthermore, when alcohol and fluid were administered to volunteers to suppress AVP secretion, all 6 subjects had undetectable AVP after drinking 4 pints of beer at a time when free water clearance was maximal (22). The third method to test whether the assay could detect low values was the osmotic stimulation of 3 patients with severe cranial diabetes insipidus. All patients had undetectable plasma AVP concentrations under basal conditions and after osmotic stimulation only one had a measurable value (0.4 pmol/l). These observations are in keeping with previous results of studies in diabetes insipidus using a different well-characterised antiserum (23).

The values of plasma AVP obtained in the patient with SIAD are consistent with his underlying pathology and the results indicate that he had random, erratic secretion of AVP in relation to plasma osmolality, suggesting a Type 1 form of SIAD (24).

We conclude that this new antiserum to AVP is extremely sensitive and specific, readily allowing measurement of plasma AVP in the physiological range on samples of small volume.

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