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## HPLC METHOD FOR QUANTIFICATION OF ARGININE CONTAINING VASOPRESSIN

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### ABSTRACT

An isocratic technique was developed for the analysis of [Arg<sup>8</sup>]- vasopressin (AVP) by reverse-phase high performance liquid chromatography (RP-HPLC) using a 220 nm UV detection, Brownlee Spheri-5 ODS column (250 x 4.6 mm, 5 μm, 100 Å), mobile phase (methanol: 0.1% aqueous trifluoroacetic acid: : 3:7), and 1.5 mL/min flow rate. The coefficients of variation (C.V.) for precision and proportionality assays were lower than 2% for all concentrations studied. The detection limit, recovery rate, and tailing factor for AVP were 50 ng/mL (signal-to-noise ratio of 3:1), 0.97 ± 0.03, and 0.99-1.1, respectively.

### INTRODUCTION

Vasopressin is synthesized in the hypothalamus and transported to the posterior pituitary for storage. It is released in response to hyperosmolality, hypovolemia, hypotension, emotional stress, posture, temperature, and many pharmacological agents.<sup>1</sup> AVP can be used to treat diabetes insipidus as well as a number of other diseases, such as hyponatremia.<sup>2,3</sup> AVP is usually referred to as antidiuretic hormone by physiologists and biochemists because it decreases

urine flow by increasing the resorption of water from the distal convoluted tubules and collecting ducts of the kidney. Several HPLC methods are now available for the analysis of AVP.<sup>4,5,6</sup> Each method has its own advantages and disadvantages.

In the method devised by Rao et al,<sup>4</sup> an isocratic high performance liquid chromatography-photodiode-array detection method is utilized for the determination of lysine- and arginine- vasopressins and oxytocin in biological samples. This method is sensitive, but it requires a specific instrument: a photodiode-array system. In the Rhodes and Boppana method, post-column fluorescence reaction was used to determine AVP in biological fluid. It is very sensitive, but the conditions used in the method, such as high post column temperature (70°C) and basic solution, will affect the stability of AVP. In short, the previously mentioned methods are sensitive and useful for the determination of AVP, but they can not guarantee the separation of AVP from its degradation products completely. Therefore, caution is necessary when using these two methods in AVP stability study. In the Paulsen et al.<sup>6</sup> method, the chromatogram provided seems to indicate that other products co-elute with AVP. Thus, in this study, we developed a simple and fast HPLC separation method for the quantitation of AVP that could be used in the stability testing of AVP in various pharmaceutical dosage and delivery systems.

## EXPERIMENTAL

### Materials

Synthetic [Arg<sup>8</sup>]-vasopressin was obtained from Sigma Chemical Company (St. Louis, Missouri). HPLC-grade methanol and trifluoroacetic acid were obtained from Fisher (Los Angeles, Tustin, CA). C<sub>18</sub> MICROSORB-MV<sup>TM</sup> column (silica 5 μm, 100 Å, 25 cm × 4.6 mm) was procured from Rainin Instrument Company, Inc. (Woburn, MA). ZORBAX C<sub>8</sub> (15 cm x 4.6 mm, 5 μm, and 100 Å ) was obtained from the DUPONT Company (Analytical Instruments Division, Wilmington, DE). Brownlee Spheri-ODS column (25cm x 4.6 mm, 5 μm, 100Å) was obtained from Alltech Associates, Inc. (Deerfield, IL). All solutions and buffers were prepared with distilled deionized water.

### Method

Hewlett Packard series 1050 liquid chromatograph (Hewlett Packard, Germany) was used. The above HPLC system consisted of a pump (HP 1050), an injector (HP 1050), a variable-wavelength UV detector (HP 1050), and a

computing integrator (HP 3396 A series). AVP and its degradation products were eluted from the columns and detected at 220 nm. The mobile phase consisted of 0.1% aqueous TFA and methanol. The injection volume was 100  $\mu$ L. Stock solution of AVP was 2.5 mg/mL in water. Test solutions were prepared by adding 20  $\mu$ L of stock solution to vials containing 0.98 mL of phosphate buffer (0.1 M, pH 2.6-8.5).

The reaction vials were then placed into a constant temperature oven at  $80 \pm 0.1^\circ\text{C}$ . Samples were removed from the oven at 72 h and the concentrations of AVP were assayed by HPLC. Standard curves were constructed from five concentrations of standard samples (1.6-50  $\mu\text{g/mL}$  in mobile phase). These samples were also used to determine the intra-day and inter-day variations of the method.

Intra-day determinations were carried out at five separate times of the day. Five standard samples were injected each time. The interval was two hours. Five values (height) for each sample and five groups of data for five standard samples were calculated after ten hours. The slope and  $R^2$  for each group of data were also calculated. Means and coefficients of variation (C.V.) were calculated from these values.

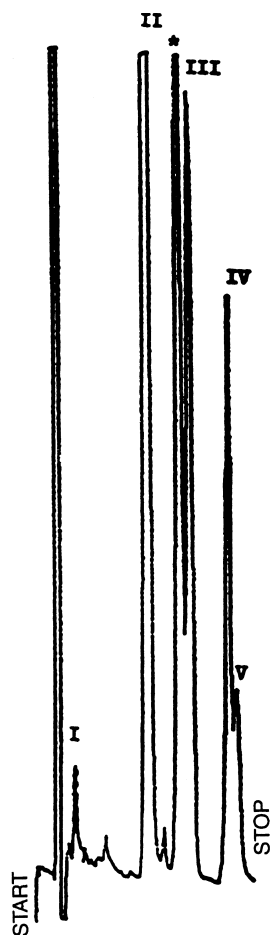
Inter-day tests were carried out at the same time of each day. The calculation method was same as that of the intra-day test. The Peak asymmetry factor was calculated by  $T=W_{0.05}/2f$  ( $W_{0.05}$  is the width of the peak at 5% height; and  $f$  is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline).

## RESULTS AND DISCUSSION

### Optimization of HPLC Conditions

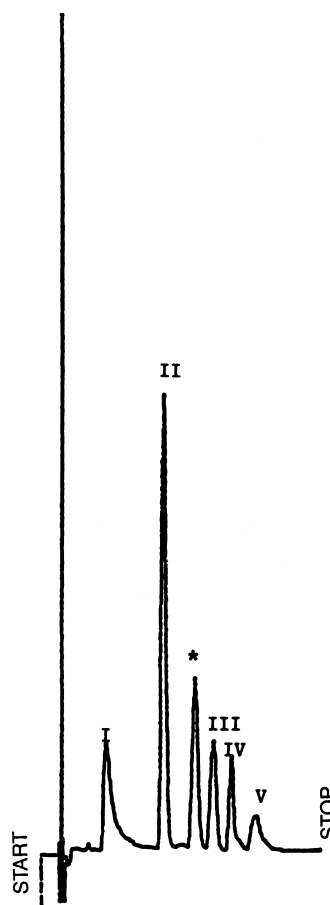
The effect of flow rates, columns, mobile phase compositions, and temperatures on the resolution of AVP and its degradation products was investigated. Methanol in combination with 0.1% aqueous TFA was used as the mobile phase. TFA can bind the amine group in arginine and reduce the protonation of the silanol group in the column which prevents tailing of the peak. Three different columns were selected in order to optimize the HPLC conditions for the separation of AVP and its degradation products.

We began with the mobile phase (methanol: 0.1% aqueous TFA : : 50:50) and changed the  $k'$  value of AVP in the  $\text{C}_{18}$  MICROSORB<sup>TM</sup> column by changing the amount of methanol in the elution liquid to restrict the  $k'$  value



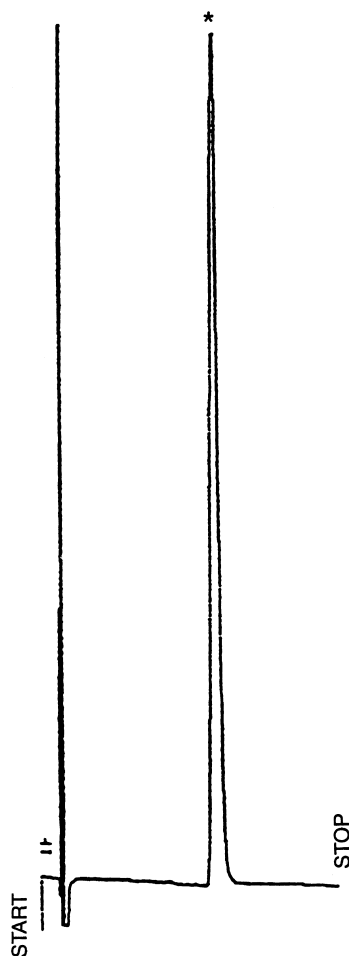
**Figure 1.** [Arg<sup>8</sup>]-Vasopressin and its degradation products in phosphate buffer (pH 2.6) stored at temperature 80°C for 72 hours (analyzed by ZORBAX C-8 column). \* is the AVP peak. I, II, III, IV, and V are degradation peaks.

within 20 at a flow rate 1.5 mL/min. The main peak of AVP was not quite separated from its degradation products and a tail occurred in the main peak. In addition, the replacement of the C<sub>18</sub> MICROSORB™ column with ZORBAX C<sub>8</sub> (15 cm × 4.6 mm) and restricting the k' value within 20 at a flow rate of 1.5 mL/min did not produce satisfactory separation (Figure 1: no complete separation between degradation product III and main peak AVP). Similar finding was obtained when the column temperature was raised to 60°C while keeping the k' value and flow rate constant. Consequently, we changed the



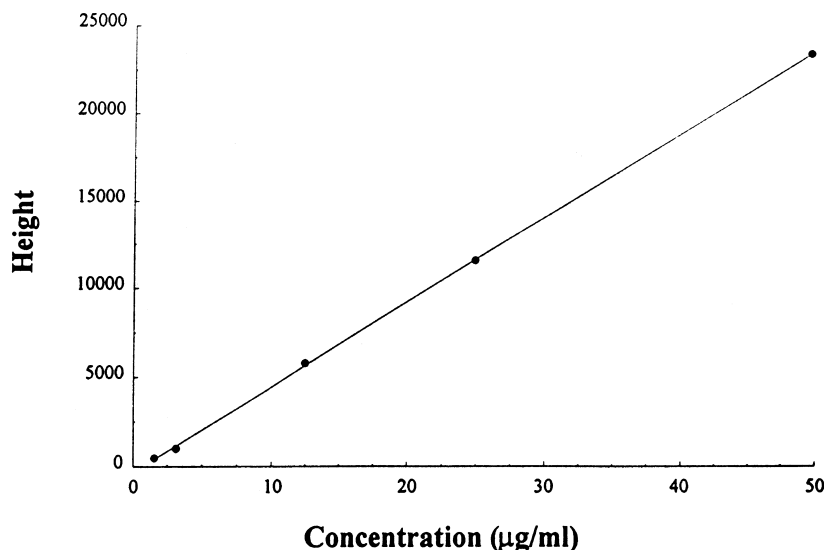
**Figure 2.** [Arg<sup>8</sup>]-Vasopressin and its degradation products in phosphate buffer (pH 2.6) stored at temperature 80°C for 72 hours (analyzed by Brownlee Spheri-5 ODS column). \* is the AVP peak. I, II, III, IV, and V are degradation peaks.

column to Brownlee Spheri-ODS column (250 x 4.6 mm, 5 μm, 100Å) and kept the  $k'$  value and flow rate as before by changing the percentage of methanol in the mobile phase. We found that the Brownlee Spheri-ODS column (25 cm x 4.6 mm, 5 μm, 100Å) had the highest resolution and selectivity which could separate AVP from its degradation products completely. The separation time obtained was within 25 min (Figure 2). The resolution increased when the flow rate of the mobile phase was decreased, but the resolution factor  $[R_s = 1.18 (t_2 - t_1) / (w_{1/2} + w_{1/2})]$  did not change much and the separation time increased ( $t_1$  and



**Figure 3.** Standard HPLC Chromatogram of [Arg<sup>8</sup>]-vasopressin in phosphate buffer (pH 2.6). \* is the AVP peak.

$t_2$  : retention time of the two adjacent peaks,  ${}_1w_{1/2}$  and  ${}_2w_{1/2}$ : peak width at half height of the peak). Thus, we determined that optimum HPLC conditions are as follows: mobile phase ( 0.1% aqueous TFA : methanol : : 7:3), Brownlee Spheri-5 ODS column (25 cm x 4.6 mm, 5  $\mu$ m, 100 $\text{\AA}$ ), flow rate (1.5 mL/min), injection volume (100  $\mu$ L, UV detection wavelength (220 nm) and column temperature 35 $^\circ$ C. We also determined the separation capability of AVP and its degradation products in each pH value using the above optimum conditions. We found that AVP could be separated from its degradation products in each pH value studied.



**Figure 4.** Standard calibration curve of [Arg<sup>8</sup>]-vasopressin in concentrations range of 1.6-50 µg/mL.

In this study, we have not characterized the degradation products. However, vasopressin structure (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH<sub>2</sub>) reveals that several reactions might occur, such as deamidation (Gln, Asn), hydrolysis (HN-CO bond), oxidation and photooxidation (Tyr, Phe), and β-elimination (Cys). Therefore, the corresponding degradation products will be produced. Since the test solution was prepared in phosphate buffer which was different from the standard solution prepared in mobile phase, possible matrix effects might exist which would affect the resolution. However, only 100 µL of test solution was injected into the column each time which was much less compared with the large amount of mobile phase. Furthermore, the polarity of both solutions was equal to or stronger than the mobile phase. Therefore, the interaction between AVP, its degradation products, and C<sub>18</sub>H<sub>36</sub> will not be affected. The peak asymmetry factors for AVP was in the range of 0.99-1.1 (Figures 2 and 3).

#### **Linearity, Detection, Reproducibility, and Recovery**

The linearity assay consisted of the determination of the same concentration range of AVP as the calibration curve (1.6- 50 µg/mL) and each concentration was analyzed five times. The peak height was linearly related to



**Table 1****Intra-Day Precision for Vasopressin Determination**

Concentration ( $\mu\text{g/mL}$ )	Height (Five Different Times)					Average	% C.V.
	(1)	(2)	(3)	(4)	(5)		
50	23591	23576	23592	23546	23535	23568	0.11
25	11491	11520	11517	11707	11604	11567.8	0.77
12.5	5799	5797	5805	5734	5730	5773	0.65
3.13	989	995	990	991	994	991.8	0.26
1.56	464	471	474	466	483	471.6	1.59
Slope	478.5	478.2	478.4	478.7	477.9	478.4	0.07
$R^2$	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	

**Table 2****Inter-Day Precision for Vasopressin Determination**

Concentration ( $\mu\text{g/mL}$ )	Height (Five Different Times)					Average	% C.V.
	(1)	(2)	(3)	(4)	(5)		
50	23591	23547	23584	23534	23567	23564.6	0.1
25	11500	11510	11513	11697	11630	11570	0.77
12.5	5800	5802	5805	5750	5740	5779.4	0.54
3.13	950	996	992	991	993	992.4	1.96
1.56	465	469	471	465	485	471	1.75
Slope	478.9	477.6	478.3	478.4	478.6	478.3	0.1
$R^2$	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	

the concentration of AVP. The equation for the straight line was  $a = 478.3b - 344.9$  ( $r^2 = 0.9999$ ) (Figure 4). The detection limit for AVP in this method, at a signal-to-noise ratio of 3:1, was found to be 50 ng/mL. The reproducibility of the method can be expressed as both the intra-day variability and the inter-day variability.

The intra-day system precision (% C.V.) for AVP in the concentrations range 1.6 - 50  $\mu\text{g/mL}$  was 0.11 - 1.59%, and the intra-day method precision (% C.V.) was 0.07% (Table 1). The inter-day system precision (% C.V.) for AVP in the same concentrations range was 0.1 - 1.96%, and the inter-day method precision (% C.V.) was 0.1% (Table 2).

A 50 µg/mL of pure peptide (AVP) was injected into the Brownlee Spheri-5 ODS column under the conditions defined and when an increase in the absorbance at 220 nm was registered, the eluate was collected manually. The volume measured was 4 mL each time. The experiment was repeated three times and the absorbance at 220 nm of the eluate was correlated to that of a known concentration (1.25 µg/mL) of the peptide dissolved in the mobile phase. The recovery of the peptide chromatographed under the conditions was  $0.97 \pm 0.03$  (mean  $\pm$  s.d).

### CONCLUSIONS

An HPLC method for quantification of AVP was developed. The method was validated and the C.V. obtained were below the maximum permitted values.

This method can be used to study the stability and for the quantification of AVP in pharmaceutical dosage and delivery systems.

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