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Isocratic high-performance liquid chromatography–photodiode-array detection method for determination of lysine- and arginine-vasopressins and oxytocin in biological samples

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ABSTRACT

A simple, isocratic, sensitive (1 ng), and specific high-performance liquid chromatographic (HPLC) method based on photodiode-array detection (PAD) is described for simultaneous quantitation of the bioactive peptides, lysine vasopressin (LVP), arginine vasopressin (AVP) and oxytocin (OXY). Acidified pig plasma and left ventricular (LV) tissue samples were first extracted with Sep-Pak C_{18} columns, and the bioactive peptides were eluted with methanol, then dried at 37°C and reconstituted with HPLC mobile phase. The bioactive peptides were separated by HPLC on a Dynamax 3009-A C_8 column with a mobile phase of 0.1% trichloroacetic acid–50 m*M* heptanesulfonic acid–30m*M* triethylamine–20% acetonitrile in water, pH 2.5 and identified with a Waters 990-PAD system (spectrum index plots in the range 200–400 nm). Standards of LVP, AVP and OXY and their mixtures showed a linear increase in the range 5 to 100 ng and were eluted at 6.1, 6.9 and 4.6 min, respectively. Spectrum analysis showed a distinct absorption peak at 280 nm, corresponding to peptide bonds. The reproducibility of the method coefficient of variation for standards is 6.9, 5.8 and 4.7% for LVP, AVP and OXY, respectively.

In plasma and tissue it is much higher: 12.9% (LV tissue) and 18.6% (plasma) for LVP. Pig plasma contains negligible amounts of AVP and OXY; LVP is much higher (0.28 ± 0.19 ng/ml). In pig tissue, LVP predominates (6.95 ng/g wet weight) compared to AVP (1.45) and OXY (1.50). Spectral analysis is necessary to identify the bioactive peptide peaks among interfering substances and to increase the sensitivity four-fold. The method described here is useful for the simultaneous determination of LVP, AVP and OXY in the nanogram range and can be extended to picogram levels by employing PAD spectral analysis techniques.

INTRODUCTION

The bioactive peptide hormones in this study are important clinical and pharmaceutical substances [1]. Lysine vasopressin (LVP) is an antidiuretic, arginine vasopressin (AVP) has antidiuretic, pressor and neurotransmitter activity [2]. Oxytocin (OXY) is used as a birth-inducing and lactation agent. The hormones are synthesized in the hypothalamus (AVP) and paraventricular nucleus (LVP) of primates [3] and transported to the posterior pituitary for storage and eventual release. Vasopressins

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are released in response to hyperosmolality, hypovolemia, hypotension, emotional stress, posture, temperature and with many pharmacological agents [4]. Vasopressin assays are clinically useful in hyponatremia, diabetes insipidus and in antidiuretic hormone as a neurotransmitter [5]. Radioimmunoassay kits for AVP measurements are available [6], but not for LVP or OXY. Pig plasma predominantly contains only LVP and its measurement is very important for elucidating its role after acute hemorrhage and during prolongation of hypovolemia and hypotension in developing swine.

We describe here a direct isocratic high-performance liquid chromatography (HPLC)–photodiode-array detection method for simultaneous determination of LVP, AVP and OXY in standard mixtures and in biological samples.

EXPERIMENTAL

Materials

HPLC-grade acetonitrile (Fisher, Springfield, NJ, U.S.A.) was used. Analytical grade chemicals were obtained from the following sources: trifluoroacetic acid (Fisher), heptanesulfonic acid (Waters PIC-B7, Milford, MA, U.S.A.), and triethyl amine (Kodak, Rochester, NY, U.S.A.). Water was purified with a Milli-RO and Milli-Q filtration system (Millipore, Bedford, MA, U.S.A.).

The HPLC system consisted of a Waters 510 HPLC pump system, coupled to a Waters 840 Data and Chromatography Control Station with a Waters U6K injector and a Waters 990 photodiode-array system, PAD (Waters Assoc., Milford, MA, U.S.A.). The column used was a Dynamax 300-A C_8, measuring 5 mm by 28 cm (Rainin Instruments, Woburn, MA, U.S.A.). Injections were made with a 25-μl Hamilton syringe (Waters Assoc.).

Methods

The mobile phase consisted of 0.1% trifluoroacetic acid–50 m*M* heptanesulfonic acid–30 m*M* triethyl amine–20% acetonitrile in water (pH adjusted to 2.5 with Na_2HPO_4) filtered through the Duropore filter (0.22 μm) (Waters Assoc.) and degassed on the day of use. The flow-rate was 1 ml/min, and the sample volume injected was 20 μl. The UV absorption spectral index and spectrum analysis were performed with the PAD technique in the range 200–400 nm.

Standards of AVP, LVP and OXY (Calbiochem, La Jolla, CA, U.S.A.) and their mixtures (1–100 ng) were used. Pig EDTA plasma (2 ml) of LV tissue (500 mg homogenized in 6 ml 0.1 *M* Tris, pH 7.4) was acidified with 200 μl of 1 *M* hydrogenchloride (pH 3.0). Extraction of biopeptides from plasma was performed using methanol washed octadecasilyl-silica (ODS-Silica) columns (Waters Assoc.). The peptides were then eluted from the column with 3 ml of methanol over a period of 3 min and with another 2 ml for 1 min. The methanol eluate was evaporated to dryness in a 37°C bath under an air jet. After complete drying, specimens were reconstituted with 200 μl of mobile phase, ready for analysis by HPLC.

RESULTS AND DISCUSSION

The spectrum index and spectrum analysis plots with a standard mixture (10 ng) of AVP, LVP, and OXY are shown in Fig. 1. Oxytocin has a retention time of 4.6

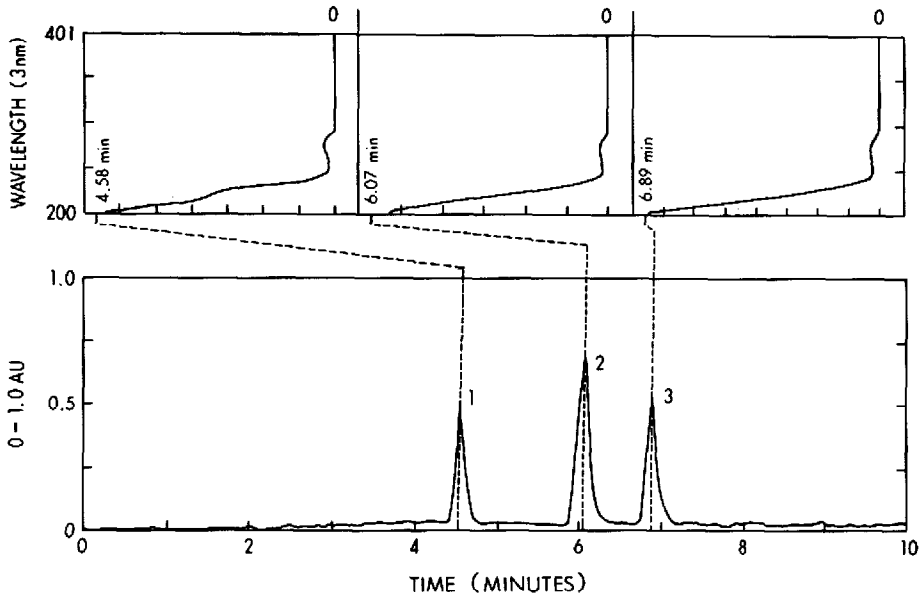

Fig. 1. Spectrum index plot of a standard (10 ng) mixture of (1) OXY (4.58 min), (2) LVP (6.07 min) and (3) AVP (6.89 min). The absorption spectra of the retention time peaks in the range 200–401 nm (inset, top).

TABLE I

PRECISION OF THE METHOD (COEFFICIENT OF VARIATION, C.V.)

Sample	Range (ng)	C.V. (%)		
		Standard (n = 15)	Pig LV tissue[a] (n = 5)	Pig plasma[a] (n = 5)
AVP	10–100	6.9	19.6	24.6
LVP	20–100	5.8	12.9	18.6
OXY	5–100	4.7	15.5	17.8

TABLE II

NORMAL VALUES IN PIG LV TISSUE AND PLASMA

Results are mean values ± S.D. (n= 5)

Sample	AVP	LVP	OXY
Pig LV tissue (ng/g wet weight)	1.45 ± 1.30	6.95 ± 4.75	1.50 ± 0.95
Pig plasma (ng/ml	0.05 ± 0.09	0.28 ± 0.19	0.10 ± 0.11

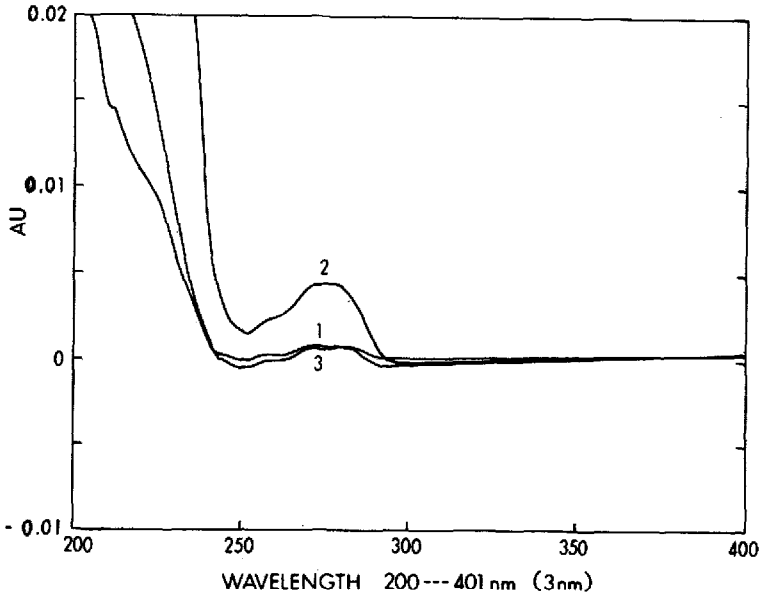

Fig. 2. Spectrum analysis plot of a standard (10 ng) mixture of (1) OXY, (2) LVP and (3) AVP (peak at 280 nm).

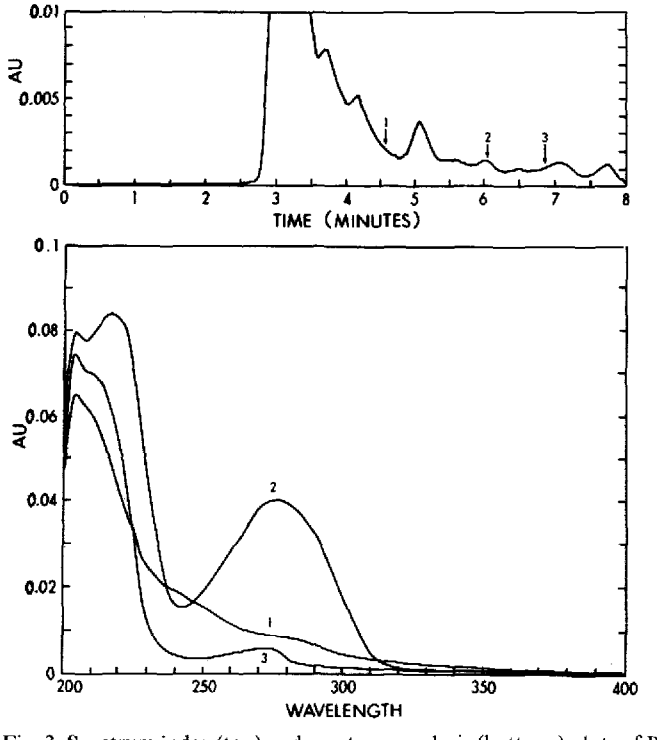

Fig. 3. Spectrum index (top) and spectrum analysis (bottom,) plots of Pig plasma. 1 = OXY (4.58 min); 2 = LVP (6.07 min); 3 = AVP (6.89 min).

min and appeared first, followed by LVP (6.1 min) and then AVP (6.9 min). All three have absorption maxima at 280 nm (top, Fig. 1). A linear increase with each standard in the range 1–100 ng was obtained, validating the method. The reproducibility of the method for duplicates ($n = 15$) is presented in Tables I and II. For standards, the C.V. values were 6.9, 5.8 and 4.7% for LVP, AVP and OXY, respectively. Inclusion of heptanesulfonic acid (ion-pairing agent) and triethyl amine (peak symmetry) and adjustment of pH to 2.5 with phosphate buffer (ion-exchange effect) gave very distinct peaks with good separation within 8 min. In the absence of the above agents (pH *ca.* 1.2), LVP and AVP were eluted at the same retention time (3.8 min) followed by OXY (5.0 min), making quantitation of LVP/AVP impractical.

Spectrum analysis of the standards (Fig. 2) shows characteristic absorption peaks at 275–280 nm for all three peptides. OXY, in addition, has a distinct additional peak at 225 nm, while the others continue to have a peak at about 200 nm.

Photodiode array spectral index analysis of pig plasma (Fig. 3) shows many small indiscrete peaks, probably from other bioactive peptides. The AVP and OXY are negligible (top, Fig. 3) and LVP (6.07 min) shows an absorption peak at 275 nm (absorbance = 0.04), corresponding to 0.26 ng/ml. This value is high compared to the levels (5–10 pg) reported by radioimmunoassay method and most of it may be due to the interfering peak at 6.5 min. Pig. LV tissue spectrum index analysis (Fig. 4) reveals

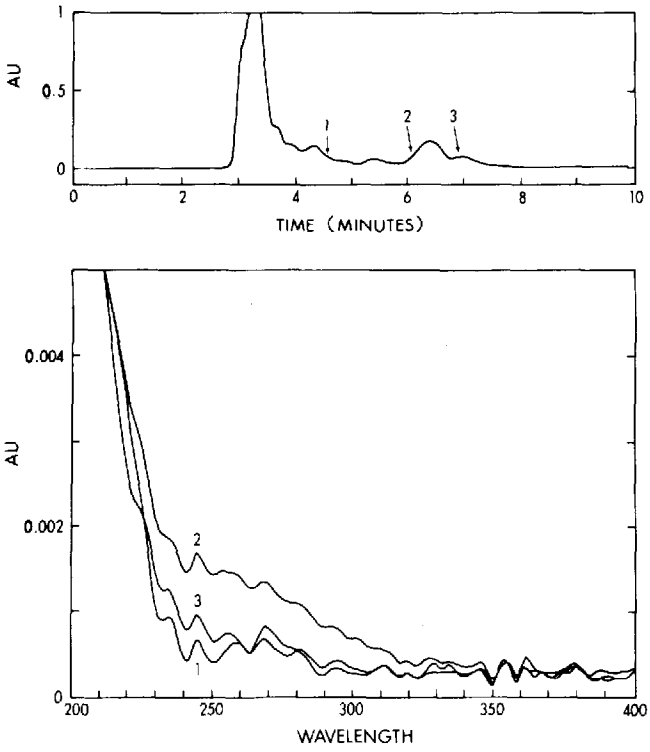

Fig. 4. Spectrum index (top) and spectrum analysis (bottom) of Pig LV tissue. 1 = OXY (4.57 min); 2 = LVP (6.06 min); 3 = AVP (6.88 min).

negligible amounts of AVP and OXY, measurable only by spectrum analysis plot (bottom, Fig. 4). AVP and OXY contents average 1.45 and 1.50 ng/g wet weight, respectively. LVP, on the other hand, is appreciable and measures 6.95 ± 4.75 ng/g wet weight.

HPLC provides a direct, sensitive (1 ng) and reproducible method for simultaneous determination of AVP, LVP, and OXY *in vitro* and in pharmacological samples. In biological samples, spectral analysis of the individual biopeptides is essential to distinguish them from interfering biopeptides and to improve sensitivity four-fold.

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