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Fluorometric assay of vasopressin and oxytocin: A general approach to the assay of peptides in tissues

(pituitary/liquid chromatography)

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ABSTRACT **A fluorometric method for the quantitative assay of vasopressin and oxytocin in individual rat pituitaries has been developed. Acid extracts of pituitaries are freed of amino acids and polyamines by passage over a copper-Sephadex column, and the peptide fraction is then labeled by reaction with fluorescamine. The resulting peptide fluorophors are separated by chromatography on a reverse-phase bonded column. Specificity of the procedure was ascertained by several criteria, including bioassay and amino-acid analysis of the eluted peptide fluorophors. The procedure serves as a model system for the assay of tissue peptides in the picomole range.**

Transmission of information by chemical agents is widespread in biological systems, whether in neural, hormonal, or immunological regulation. It is becoming evident that a large number of these chemical transmitters are peptides in the molecular weight range of 300–10,000. Active peptides have generally been discovered through their biological activity, and today, even in those instances where synthesis has been achieved, they are assayed by biological or immunological methods. Thus far, bioassay and immunoassay have been the only means with which to achieve the specificity and sensitivity required. While such assays are useful, they do not entirely substitute for specific chemical assay.

With the introduction of fluorescamine (1) and the automated instrumentation and technology for its use (2, 3), it has become possible to develop procedures for the specific and quantitative assay of individual peptides in the picomole range. Details of the assay of oxytocin and vasopressin in the extracts of rat pituitary glands are presented as an example of the general applicability of the procedure to the assay of peptides of biological interest.

MATERIALS AND METHODS

Male Wistar rats (200–250 g) were obtained from Marland Farms (Hewitt, N.J.). Microhomogenizers were from Kontes Glass (Vineland, N.J.). Peptides for use as standards were from Chemical Dynamics (South Plainfield, N.J.). Polypropylene tubes (1.5 ml) and their centrifuge were obtained from Brinkmann Instruments (Westbury, N.Y.). Hoffmann-La Roche Inc. (Nutley, N.J.) kindly supplied the fluorescamine, which was dissolved in Spectranalyzed acetone (Fisher Scientific, Springfield, N.J.). Water was purified by a system from Hydro Service and Supplies (Durham, N.C.), and was free of electrolytes (resistance greater than 10^6 ohms).

Prepacked microparticulate chromatography columns, Partisil ODS and SCX, were obtained from Whatman (Clifton, N.J.). The pumping system used was a 5000 psi (35 MPa) Milton Roy mini-pump (Lab Data Control, Riviera Beach, Fla). Samples were applied with a Valco 7000 psi injection valve (Valco Instruments, Houston, Tex.). The solvent gradient was programmed by an LKB Gradient Mixer (LKB Instruments, Hicksville, N.Y.). Fluorescence was measured by a Fluoromicrophotometer (American Instrument, Silver Spring, Md.) equipped with a modified 25 μl flow cell and appropriate filters. Additionally, a piece of plastic polarizing material no. 70, 420 (Edmund Scientific, Barrington, N.J.) was placed over each slit of the fluorometer, one at right angle to the other, to reduce light scattering. A strip chart recorder (Beckman Instrument, Palo Alto, Calif.) was used to plot fluorescence.

Peptides were hydrolyzed under reduced pressure in ampules containing constant boiling HCl with 0.1% thioglycollic acid (4) at 110° for 24 hr. The hydrolysates were assayed with an analyzer designed for fluorescamine (2). The sensitivity of this system is in the picomole range.

The bioassay of vasopressin was carried out using the rat pressor method (5), except that samples were injected into the jugular instead of the femoral vein.

RESULTS

Peptides for use as standards

A major problem in establishing the assay of any peptide appears to be the difficulty in obtaining a pure sample to be used as a standard. Even with oxytocin and vasopressin, where synthesis was carried out many years ago, pure samples are not readily available, and even the purest material from commercial sources contains other components. Purification and verification of purity of the standards are requisites for assay.

Commercially obtained vasopressin and oxytocin were purified by chromatography on a 25 × 0.46 cm column of Partisil SCX (a cation exchange resin). Elution was at room temperature at 8.0 ml/hr, utilizing a gradient from 0.005 M pyridine-acetate to 0.5 M pyridine-acetate, pH 3.0 and 5.0, respectively. Details on the use of this column for chromatography of free peptides will be presented elsewhere. The peptides in the column eluate were detected by the fluorescamine reaction, using a discontinuous sampling valve (6). Homogeneity of each peak was confirmed by chromatography of the fluorescamine derivative on Partisil ODS (see below). Amino-acid analysis of each peak confirmed the identity and was used for quantitation of the primary standards.

Extraction of peptides from tissues

The procedure described by Sachs *et al.* (7) was found to be satisfactory for oxytocin and vasopressin. Rats were killed by

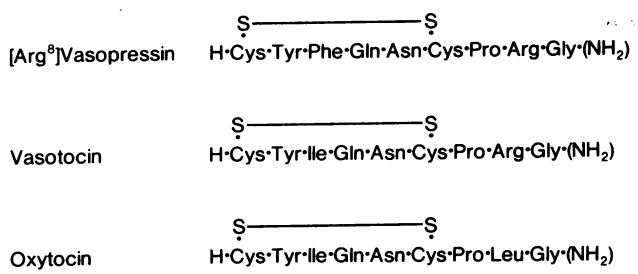

FIG. 1. Structures of [Arg^8]vasopressin, vasotocin, and oxytocin.

decapitation with a guillotine, and the posterior lobes of the pituitaries were removed within 1 min.*

The following steps were carried out at 0–4°. An individual pituitary was homogenized by hand in a microhomogenizer in 70 μl of a solution composed of 0.02 M HCl, 0.2 M acetic acid, and 0.01% thiodiglycol (HCl-HOAc). The homogenization solution also contained 1.0 nmole of an analog, vasotocin, as an internal standard to correct for sampling and small losses incurred at each step of the procedure. The structures of the three peptides are shown in Fig. 1. Vasotocin was chosen for this purpose because, although its structure is similar to the other two, its fluorescamine derivative elutes as a separate peak on subsequent reverse-phase chromatography. Furthermore, it is not present in rat pituitary extracts.

The homogenate was transferred to a 1.5 ml polypropylene centrifuge tube. The homogenizer and pestle were then rinsed with two 70 μl portions of HCl-HOAc, which were then added to the tube.† After centrifugation at 6000 rpm for 3 min, 70 μl of 40% trichloroacetic acid, containing 0.01% thiodiglycol, was added and the sample was shaken gently. After 40 min the tube was centrifuged again and the supernatant, as well as two 50 μl rinses of the precipitate with HCl-HOAc, was transferred to another centrifuge tube. The combined extracts were then shaken with three 1 ml portions of ethyl ether, which had been presaturated with 0.01 M ferrous chloride, to remove the trichloroacetic acid as well as lipids and lipoamines. Residual ether was removed by blowing nitrogen over the sample for 1 min at room temperature.‡

Separation of peptides from α-amino acids

The use of copper-Sephadex to remove α-amino acids from peptide solutions is well documented (8). Additionally, this resin was found to retard lipoamines and polyamines while allowing peptides and ω-amino acids to pass through. However, amino-acid analysis of the eluted peptides revealed that certain amino acids, such as cystine and tyrosine, were oxidized. This problem was solved by treatment of the resin with thiodiglycol, and the addition of 0.01% thiodiglycol to all aqueous solutions.

Prior to passage over copper-Sephadex, the ether extracted tissue sample (about 300 μl in volume) was adjusted to pH 11.0 (indicator paper) by adding microliter quantities of KOH. Copper-Sephadex was prepared according to the method of Fazakerly and Best (8). It was washed with 0.01 M sodium tetraborate titrated to pH 11.0 with KOH, and made up to contain 0.1% thiodiglycol. A 3 × 0.7 cm column was packed in a siliconized glass tube, and polyethylene discs were used to sandwich the resin bed as previously described (3). After the column was washed with several bed volumes of the borate buffer, the sample was allowed to drain into it. Peptides were eluted in the first 1.0 ml of the effluent, which was collected in a 1.5-ml plastic centrifuge tube.

Preparation of fluorescamine derivatives

Peptides react with fluorescamine through their free amino groups to yield the corresponding derivatives. The reaction with peptides is maximal at about pH 7, whereas reaction with α-amino acids is best at pH values in the range of 8–9. The reaction was therefore carried out at pH 7. It was also necessary to add EDTA before the reaction because traces of free copper from the copper-Sephadex column were found to quench the fluorescence. Accordingly, the 1 ml of the borate buffer eluted from each column was neutralized by the addition of 0.125 ml of 0.5 M KH_2PO_4 in 0.02 M disodium EDTA. An aliquot of the neutralized extract, equivalent to about 350 μl, was transferred to a 1.5 ml polypropylene tube and treated with 200 μl of fluorescamine dissolved in acetone (20 mg/100 ml). While it has been shown that under similar reaction conditions 80–90% of a peptide is rapidly converted to a single fluorescent derivative, 10 min was allowed to insure complete hydrolysis of excess reagent. The extent of fluorophor formation with vasopressin was determined in the following manner. After vasopressin was reacted with fluorescamine, an aliquot of the reaction mixture was hydrolyzed and subjected to amino-acid analysis. Another aliquot, when chromatographed on a reverse-phase column (see below), yielded a single fluorescent peak. The material in the entire peak was collected, hydrolyzed, and subjected to amino-acid analysis. Since recovery of peptide fluorophors from the columns is quantitative (see below), from a comparison of the analyses it was calculated that 89 ± 5% (duplicate samples) of the vasopressin was converted to the fluorophor.

Chromatography of the fluorescamine derivatives

To the solution containing the fluorescamine derivatives prepared above (about 550 μl) was added 650 μl of the 0.03% ammonium formate containing 0.01% thiodiglycol. It is important that the acetone content be less than 15%. One milliliter of the solution was then applied to a Partisil ODS (reverse-phase) column which had been pre-equilibrated for 10 min with 15% acetone containing 0.03% ammonium formate and 0.01% thiodiglycol.

A 55 min linear gradient from 15 to 50% acetone, with both solutions containing ammonium formate and thiodiglycol at the above concentration, at a flow rate of 0.25 ml/min was used to elute the peptide fluorophors. After each run the column was washed with 80% acetone for 5 min, and then re-equilibrated. Reverse-phase chromatography of an extract representing approximately 25% of a single rat posterior pituitary gland is shown in Fig. 2. The early eluting material most probably contains smaller peptides and ω-amino acids. It should be noted that the area of the chromatogram where vasotocin elutes is relatively free of interfering material. The peak heights of vasopressin and oxytocin, relative to vasotocin, in duplicate pituitary aliquots agreed within ±4%.

* Delaying dissection and homogenization for 5 min after decapitation did not significantly alter the pituitary vasopressin and oxytocin levels.

† Each rinse was monitored for vasopressin by the bioassay procedure. Only the first two washes contained significant amounts of pressor activity.

‡ The nitrogen was passed through a gas scrubbing bottle which contained 3 M H_2SO_4.

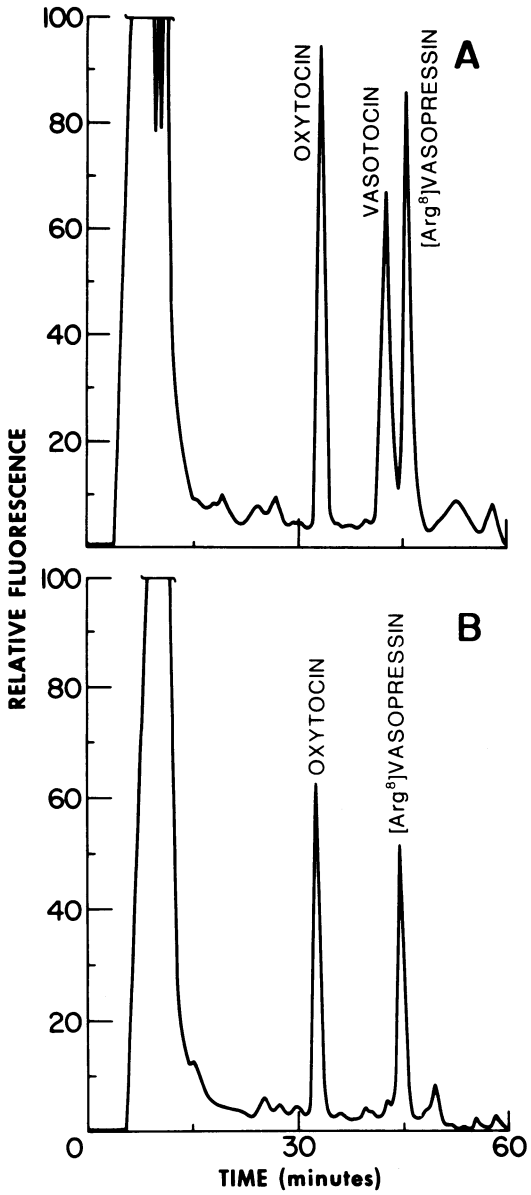

FIG. 2. A and B show a chromatogram of an extract representing 25% of a rat pituitary with and without the addition of the internal standard vasotocin.

Recovery of peptides

It was shown that the recovery of the fluorophors of oxytocin and vasopressin from the chromatographic columns was quantitative. When aqueous solutions of the two peptides were carried through the entire procedure, recoveries were 98% for each when corrected for sampling and completeness of reaction with fluorescamine (see above). The absolute recovery of vasotocin added to pituitary samples was approximately 90%.

Sensitivity and proportionality

No buffer artifacts or baseline fluctuations were observed when chromatography was performed without injecting a sample onto the column. When fluorescamine treated blanks were passed through the column a number of small peaks appeared, which represented trace contamination of amines in the reagents. Chromatography of 15 pmol of synthetic vasopressin and oxytocin fluorophors yielded peaks with signal to noise ratios (peak height to baseline fluctuation) of about 15:1. When extracts of pituitaries were reacted with fluorescamine and chromatographed, an elevation in baseline was observed. This did not interfere with quantification (Fig. 2), but did lower the sensitivity to some extent. Nevertheless, aliquots representing less than 20 pmol gave peak heights at least five times greater than the baseline elevation.

Under the experimental conditions described above, peak heights were linear and proportional to peptide concentration from 10 to 500 pmol for both peptides. The concentrations of oxytocin and vasopressin in both standards and tissue samples were calculated from the ratios of their peak heights to that of vasotocin.

The addition of as much as 25 nmol of alanylalanine to a mixture of vasotocin and vasopressin, prior to reaction with fluorescamine, did not diminish the peak heights of the two nonapeptides nor did it alter their retention times. This indicates that the reagent is present in excess, and that large amounts of other fluorescamine reacting material in tissue extracts do not interfere.

Specificity

The material in the peaks appearing on the chromatogram of a rat pituitary extract (Fig. 2) were identified as vasopressin and oxytocin, respectively, by the following criteria: (*i*) comparison of retention times to standards; (*ii*) superimposition of the standard peptide upon its presumptive pituitary peak; (*iii*) amino-acid analysis of the pituitary derived vasopressin and oxytocin fluorophors; and (for vasopressin only) (*iv*) comparison of chemical and biological assay on aliquots of the same sample.

When authentic vasopressin was added to a pituitary extract, there was a corresponding increase in the height of the peak eluted at 45 min, with no change in peak symmetry. The same result was obtained when oxytocin was superimposed upon its presumptive pituitary peak (elution time 32 min). The retention times of the pituitary vasopressin and oxytocin peaks were always found to be identical to those of standards. Variation from run to run was no greater than for standards (for vasopressin and oxytocin ±4%). The ratio of each retention time to that of vasotocin was far smaller (for either peptide ±1%).

To establish criteria *iii* and *iv*, several rat pituitaries were homogenized and carried through the extraction and copper-Sephadex steps without the addition of vasotocin. Aliquots were treated with fluorescamine, subjected to reverse-phase chromatography, and the fluorometer effluent corresponding to the oxytocin and vasopressin peaks was collected. The peaks were then taken to dryness, hydrolyzed, and subjected to amino-acid analysis using the fluorescamine procedure (2). Appreciable quantities of amino acids were found in addition to the expected residues of each peptide, this contamination being related to the observed elevated baseline. However, on rechromatography, each pituitary peptide fluorophor yielded only one fluorescent peak, which when collected and hydrolyzed, gave the correct amino-acid composition when corrected for buffer blank (Table 1). Cysteine was not determined for two reasons. The cysteine residue at the amino terminus forms the fluorescamine derivative and it is not released on acid hydrolysis. Although the second cysteine residue was detectable, problems in its quantification in the presence of thioglycollic acid have been encountered (4).

Other aliquots were taken from the pituitary extract to compare the chemical assay with the biological pressor assay

Table 1. Amino-acid analyses of pituitary derived peptide fluorophors

Amino acid	Vasopressin, pmol		Oxytocin, pmol		Reagent blank, pmol	
Aspartate	318	289	318	291	36	42
Threonine	0	0	30	30	0	0
Serine	19	6	12	19	39	42
Glutamate	329	312	361	334	54	42
Proline	344	347	359	349	0	0
Glycine	342	303	353	334	80	67
Alanine	0	0	0	0	0	0
Cystine	—	—	—	—	—	—
Valine	0	0	0	0	0	0
Methionine	0	0	0	0	0	0
Isoleucine	0	0	347	339	0	0
Leucine	0	0	368	358	0	13
Tyrosine	347	304	329	340	0	0
Phenylalanine	328	292	0	0	0	0
Histidine	0	0	0	0	0	0
Lysine	0	0	0	0	0	0
Arginine	335	297	0	0	0	0
Average ± a.d.	321 ± 17		341 ± 15			

Fluorescamine derivatives of the peptides in posterior pituitary extracts were resolved on the reverse-phase column. The material in the peaks appearing at the oxytocin and vasopressin positions was collected separately and rechromatographed on the same column. After collection of the material in each peak, the solvent was evaporated and acid hydrolysis was performed. Duplicate analyses were done on each hydrolysate. During the blank run (no sample applied to the column), solvent was collected at the oxytocin and vasopressin positions. The amino-acid analyses of each peptide have been corrected for the reagent blank. Values reported as zero were below the limit of measurement (5–10 pmol). Cystine was not assayed. a.d., average deviation.

(Table 2). Vasotocin was added to those samples used for chemical assay prior to fluorophor formation. The same vasopressin standard was used in both assays. The two values agree within the experimental errors of the procedures. The slightly higher value obtained in the biological assay, if significant, can be accounted for by the presence of oxytocin, which possesses a small degree of pressor activity (9).

Determination of vasopressin and oxytocin in rat pituitary

The vasopressin and oxytocin contents of several posterior pituitaries obtained from male rats are presented in Table 3. It may be noted that for each peptide the variation from animal to animal is about 15% (average deviation). Of interest is the fact that equivalent amounts of vasopressin and oxytocin were found in each pituitary gland.

DISCUSSION

As a general procedure, the prelabeling technique with fluorescamine offers many advantages over methods in which a fluorogenic reagent is added to the column effluent. In the latter case sensitivity is limited by the additions of buffer and reagent after elution, which dilute the column effluent; by the additive noise of three pumps, causing slight but persistent baseline fluctuations; and by amine contaminants in the buffers and reagents. By prelabeling the sample the column effluent can enter the fluorometer directly. The need for stringently amine-free solvents is minimized, as the fluorescamine is reacted with the sample in a small volume. Additionally, the instrument required for the method is rather simple, using only one pump.

Table 2. Vasopressin content (pmol of vasopressin/μl) of a pituitary extract determined by biological and chemical assay

Pressor assay	Fluorescamine assay
2.91	2.16
2.39	2.20

Each value of pressor activity was based on a four point assay (5). The same standard was used for both assays.

Table 3. Assay of vasopressin and oxytocin (pmol/pituitary) in rat posterior pituitaries

Rat	Vasopressin	Oxytocin	Molar ratio (vasopressin/oxytocin)
1	752	721	1.04
2	928	844	1.10
3	740	658	1.12
4	752	774	0.97
5	1176	1034	1.14
6	928	1093	0.84
Average ± a.d.	879 ± 132	854 ± 145	1.04 ± 0.09

The assay described here can achieve specificity (which can be verified in other systems), yet has the potential of resolving and identifying other compounds in the sample. Since all peptide components that react with fluorescamine (any peptide with a free NH_2-terminal or ϵ-amino group) can be resolved, experimentally induced changes in these peptide patterns can be analyzed. Additionally, quantification can be obtained through the addition of an internal standard at the beginning of the extraction procedure. The same procedure, modified with respect to the reverse-phase chromatography, can be applied to other peptide assays. It has already been applied to the assay of carnosine (β-alanyl-L-histidine) in the olfactory bulb[§].

While these studies were under way, another fluorogenic reagent [2-methoxy-2,4-diphenyl-3(2*H*)-furanone], structurally similar to fluorescamine, became available (10). The latter offers certain advantages: more stability at extremes of pH, the production of a more hydrophobic derivative which allows the resolution of smaller peptides and ω-amino acids, and the recovery of the NH_2-terminal amino acid after acid hydrolysis.[¶] It would appear that this reagent may become the reagent of choice for the fluorescent derivatization assay of peptides and other amines.

Considering the sensitivity of the procedure, it is surprising how little caution is required. Reasonable care must be taken with the water, buffers, and solvents. However, their purity can be readily determined (see above). To further prevent contamination we routinely use disposable surgical gloves during the handling of samples. Greater caution is required when the fluorescamine derivatives are collected for hydrolysis and amino-acid analysis. Tubes washed with dilute hydrochloric acid must be used for sample collection and drying, and the drying apparatus should be thoroughly cleaned between samples. We have found it beneficial to prepare new acetone buffers for any experiment in which we plan to collect the fluorometer effluent for amino-acid

[§] S. Stein and F. L. Margolis, unpublished results.
[¶] E. Mendez, personal communication.

analysis. Another possible source of contamination that can limit sensitivity is the hydrochloric acid used in hydrolysis (11).

The values obtained for oxytocin and vasopressin in rat pituitaries are of the same order of magnitude as previously reported (12). However, in our studies both the biological and chemical assays were based on standards of demonstrated purity. Such standards have not been generally available. Considering the unavailability of standards and the limitations of bioassay, it may be necessary to repeat many of the physiological studies previously performed on these peptides using this procedure. Preliminary studies from our laboratory show that when rats are deprived of water the pituitary vasopressin level falls. This is expected since vasopressin is the antidiuretic hormone. However, we have found that the oxytocin levels fall to the same extent. We are currently using this methodology to study physiological and pharmacological factors that influence the biosynthesis of these two peptides.

Note Added in Proof. The chromatographic separations of the peptide-fluorophors were obtained on reverse-phase columns with an efficiency of 6000 theoretical plates per quarter-meter (as determined by the manufacturer). The performance of these columns deteriorates after several months of usage.

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