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Determination of oxytocin in biological samples by isocratic high-performance liquid chromatography with coulometric detection using C_{18} solid-phase extraction and polyclonal antibody-based immunoaffinity column purification

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Abstract

A specific high-performance liquid chromatographic (HPLC) method is described for the reliable quantitation of oxytocin using culture media supernatants. The procedure employs solid-phase extraction, antibody-based immunoaffinity purification and isocratic HPLC with dual channel coulometric detection (ED). The lower limit of detection for this cyclic nonapeptide was 40 pg (40 fmol). Due to its relative simplicity, specificity and precision, the HPLC–ED of oxytocin is an accurate and attractive alternative to many existing quantitative methods.

1. Introduction

Until the early 1980s, the hypothalamus had been considered the exclusive source of oxytocin, a peptide hormone (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2) classically recognized to be associated with parturition and milk ejection. The identification of a non-neural source of oxytocin secreted in significant amounts from the ovarian corpus luteum of ruminants [1] was followed by the demonstration of an oxytocin-like peptide within the testes [2]. It is now clear that oxytocin has several additional functions in these and other peripheral tissues (for review see ref. 3). Because of the importance of this cyclic nonapeptide in mammalian reproductive biology a great deal of attention is focused on its quantitative measurement.

Quantitation of oxytocin using bioassays involving chicken blood pressure, rat uterus contraction and intramammary pressure often lack precision. These techniques are technically difficult and labor intensive. Radioimmunoassay (RIA) is the general method of choice for measuring both oxytocin and vasopressin, but since the sequence of these two nonapeptides differs by only two amino acids, the production of absolutely specific antibodies poses some potential difficulties. The recently developed very specific monoclonal antibodies (mAb) for oxytocin [4,5] have proven to be powerful immunologic tools that are sensitive (< 10 pg) and

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allow a large sample throughput. However, the development of radioimmunoassays is also rather time-consuming, and the assays are expensive in operation and particularly sensitive to interference by: (1) antibody binding by divalent cations (*e.g.* Ca^{2+}, Mg^{2+}), (2) high salt concentrations, (3) pH variations, and (4) non-specific binding to other macromolecules [6]. Many of these interfering substances are commonly used or present in culture media and biological samples.

High-performance liquid chromatography (HPLC) is an alternative approach that can remove the interfering substances that often compromise the precision of RIAs if the proper on-line detector is chosen. Ultraviolet (UV) absorbance and fluorescence detectors lack the sensitivity needed to measure the low levels of oxytocin present in most biological samples. However, the recent development of more advanced dual channel coulometric detectors (ED) which readily oxidize electroactive amino acids (*i.e.* tyrosine and tryptophan) at the upstream electrode and enhance detection at the downstream electrode, has resulted in quantitative measurements nearly fivefold more sensitive than those previously obtained using dated amperometric and coulometric detectors.

The purpose of the present study was to combine the inherent advantages of three modern technologies including solid-phase extraction, antibody-based immunoaffinity column purification and dual channel coulometric detection into one method to significantly enhance the quantitative measurement of the nonapeptide hormone oxytocin without the use of radioisotopes.

2. Materials and methods

2.1. Reagents

Oxytocin (lyophilized solid) was purchased from Calbiochem (San Diego, CA, USA). Glycine (free base), sodium chloride, anti-rabbit IgG (whole molecule) agarose 5-ml column and CNBr-activated Sepharose 4B were acquired from Sigma Chemical Company (St. Louis, MO, USA). HPLC reagent grade methanol (Burdick and Jackson Brand High Purity Solvents) was obtained from Baxter Healthcare Corporation (Muskegon, MI, USA). American Chemical Society (ACS) grade dibasic sodium phosphate (Na_2HPO_4), ACS reagent grade hydrochloric acid (HCl), HPLC grade glacial acetic acid (CH_3COOH), HPLC grade orthophosphoric acid (85% H_3PO_4) and Gelman 13-mm disposable 0.45-μm nylon Acrodisc syringe filters were purchased from Fisher Scientific (Norcross, GA, USA). Sep-Pak reversed-phase C_{18} cartridges (octadecylsilyl modified silica gel) were procured from Waters Associates (Milford, MA, USA). Solid-phase extraction (SPE) 3-ml C_{18} disposable columns with octadecylsilane (ODS) bonded to silica gel were procured from J.T. Baker Chemical Company (Phillipsburg, NJ, USA). Dulbecco phosphate buffered saline (PBS) tablets were acquired from Unipath Company (Oxoid Division, Ogdensburg, NY, USA). Unmodified Sepharose 4B was a gift kindly supplied by Pharmacia LKB Biotechnology (Piscataway, NJ, USA). Unpurified oxytocin antibody (Ab) was kindly donated by Dr. Dieter Schams (Technical University of Munich, Germany).

2.2. Water purification

Type 1 reagent grade water meeting the National Committee for Clinical Laboratory Standards (NCCLS) specifications with respect to specific conductance (<0.1 $M\Omega^{-1}$ cm^3), specific resistance (>10.0 $M\Omega/cm^3$), silicate (<0.05 mg/l) and bacterial contamination (<10.0 colony forming units) was prepared by a multistep procedure using a Vaponics Model VSS-30TI multiple-effect Thermevap distillation system with a condensate feedback purifier (Vaponics, Plymouth, MA, USA). In this process, water is removed from the impurities rather than removing impurities from the water.

Any remaining trace organics still present in the dispensed ultrapure distilled water were removed by post-filtration through a Sep-Pak C_{18} reversed-phase solid-phase extraction cartridge using vacuum aspiration. The resulting ultrapure

water used in the mobile phase preparation was of at least 15.0 MΩ/cm^3 quality. The most sensitive instrumentation or procedure is only as good as the reagent used.

2.3. Purification of oxytocin antibody

An anti-rabbit IgG (whole molecule) agarose 5-ml column previously stored at 4°C was preconditioned by flushing with 20 ml of 0.05 *M* phosphate buffered saline, pH 7.3 (PBS). Thirteen milligrams of an unpurified lyophilized oxytocin antibody [7] was diluted with 3 ml of PBS and applied to the anti-rabbit IgG agarose column and allowed to bind for 5 min at 22°C. The first 3 ml break-through fraction was collected and applied once again for a second pass through the anti-rabbit IgG column. Following a column wash with 10 ml of PBS, the bound oxytocin Ab was stripped from the anti-rabbit IgG column with 20 ml of a 0.1 *M* glycine–0.15 *M* sodium chloride buffer (pH 2.4). The column was rejuvenated with 10 ml of PBS and stored at 4°C with 5 ml of PBS containing sodium azide. The collected strip fraction (20 ml) was added in equal aliquots (10 ml) to two different Spectra/Por 2 (Spectrum, Houston, TX, USA) regenerated cellulose membranes with a molecular mass cut-off of 12 000–14 000 and dialyzed against water for 24 h at 4°C to remove buffer salts. Following dialysis, the dialysates were carefully transferred into separate 50-ml polypropylene test tubes, frozen slanted at −70°C for 24 h and subsequently lyophilized under vacuum using the Labconco Model 75034 bench top freeze dryer (Kansas City, MO, USA) for 24 h to remove water.

2.4. Preparation of antibody-based columns

The immunoaffinity matrix was prepared by covalently linking *ca.* 1 mg of a purified oxytocin Ab to 0.2 g of CNBr-activated Sepharose 4B. The antibody columns (1–2 ml bed volume) were prepared by packing 1.0 × 10 cm Econo-Columns (Bio-Rad, Hercules, CA, USA) with a mixture of antibody-conjugated Sepharose 4B (0.2 parts) and unmodified Sepharose 4B (0.8 parts). Each prepared column contained a 2-ml bed volume capable of binding at least 1 nmol of an authentic oxytocin standard.

2.5. Solid-phase extraction of samples

Oxytocin was extracted from culture media supernatants using the methods of Schams [7] as modified by Padayachi *et al.* [8] and described by Yasuda *et al.* [4]. Briefly, 500 μl of 50 m*M* HCl was added to 1 ml of each sample supernatant (pH 7.3 to 7.5) previously stored at −20°C, vortex-mixed briefly and centrifuged in an Eppendorf 5415 microcentrifuge (Brinkmann Instruments, Westbury, NY) at 1000 *g* for 5 min. The resulting supernatants were prepared for HPLC analysis by loading on to 3-ml SPE C_{18} columns which were fitted with 0.45-μm nylon Acrodisc syringe filters distally. All SPE columns were first preconditioned by washing with 1 column volume of HPLC reagent grade methanol followed by 2 column volumes of ultrapure polished water. Individual acidified supernatant samples diluted 1:1 (v/v) with PBS were then applied and aspirated slowly through individual SPE columns. Following sample addition, the extraction columns were washed initially with 1 column volume ultrapure water followed by 2 column volumes of a 1.5% acetic acid solution (pH 4.8) prepared in water. The samples were again washed with 1 column volume of polished water then air dried under vacuum for 5 min. Individual samples were eluted from the SPE columns with a 1.5-ml aliquot of methanol. Samples were then concentrated under vacuum in conical polypropylene 1.5-ml vials using a Savant refrigerated SpeedVac concentrator (Farmingdale, NY, USA). Recovery of oxytocin following solid-phase extraction is in the range 99–117% [8,4]. The SPE columns were regenerated with 1 column volume of methanol and can be used at least 5 times without substantial loss of extraction efficiency.

2.6. Antibody-based purification of samples

Concentrated samples were reconstituted with 1 ml of water and then applied individually to

the immunoaffinity column at 4°C. This temperature reportedly favors nonequilibrium binding and reduces cross-reactivity [9]. The immunoaffinity column was washed sequentially under vacuum with 2 column volumes of each of the following refrigerated reagents: (1) polished water, (2) 1 *M* NaCl, and (3) polished water followed by 1 column volume of refrigerated acetonitrile. Individual samples were eluted from the immunoaffinity column with a 1.5-ml aliquot of methanol. Acetonitrile normally has a higher elution efficiency on reversed-phase materials compared with methanol. However, methanol was more efficient in releasing antibody-bound oxytocin. This may be attributable to its superiority in suppressing hydrophilic interactions which may be responsible for immunoaffinity binding. Thus, methanol exhibits an effect similar to that observed in the chromatography of polar solutes on reversed-phase materials where the influence of residual silanol groups can be reduced more effectively than with acetonitrile.

The immunoaffinity column was regenerated with 1 column volume of methanol and can be used at least 25 times without substantial deterioration of binding efficiency. Eluted samples were again concentrated under vacuum in conical polypropylene 1.5-ml vials and reconstituted with 200 μl of mobile phase buffer (dibasic 20 m*M* sodium phosphate buffer pH 5.7–methanol, 60:40, v/v). Twenty microliters of each sample were injected onto the HPLC chromatography column in duplicate for oxytocin quantification.

2.7. HPLC determination of oxytocin

HPLC was performed on a Beckman 344 modular system (Beckman Instruments, San Ramon, CA, USA) consisting of two Model 110b solvent delivery modules, a Model 340 manual sample injector with a 20-μl loop and a Model 235 column heater connected in series with an ESA Model 5200 Coulochem II multielectrode electrochemical detector (Environmental Sciences Association, Bedford, MA, USA) equipped with a Model 5010 analytical cell (porous graphite). Data were digitized by a Beckman 406 analog interface and processed by Beckman analytical series System Gold data acquisition software on a Tandy 3000 NL computer. Both channels of the analog interface were connected to the dual channel coulometric detector to monitor a dual electrode transducer at an applied cell voltage of 525 mV at the screening electrode and 775 mV at the analysis electrode with an overall sensitivity of 100 nA and 50 nA, respectively.

The signal-to-noise ratio recorded from the electrochemical detector was maximized by employing precolumn extraction and purification of samples, using ultrapure solvents including deionized double distilled water, controlling the detector threshold and data acquisition rates, and squelching stray ambient electrical pulses through the use of a conditioned power supply. Sensitivity was maximized by: (1) passivating the metal components of the HPLC system initially with 6 *M* HNO_3, (2) utilizing a pulseless dampening solvent delivery system, (3) minimizing temperature fluctuations through the use of a column heater, and (4) using low applied cell potentials. Detection sensitivity was increased at the downstream analysis electrode after removal of interfering compounds at the upstream screening electrode. Detection of the oxytocin nonapeptide has previously been determined to be sensitive to the 40-fmol level.

High-performance liquid chromatography was performed isocratically at a column temperature of 30°C using System Gold software as the controller for the HPLC system. The eluent consisted of a pre-mixed, 0.22-μm vacuum filtered and degassed 60% dibasic 20 m*M* sodium phosphate (Na_2HPO_4) buffer, brought to pH 5.7 with 3.3 *M* HPLC grade phosphoric acid (H_3PO_4), and 40% methanol (v/v) at a flow-rate of 0.5 ml/min. The total run time was 12 min with a 3-min column clean-up period allowed between successive chromatographic runs. The reversed-phase ODS C_{18} Beckman Ultrasphere XL chromatography column (70 × 4.6 mm I.D., 3 μm particle size) and the in-line electrochemical detector were preconditioned by allowing the eluent to recirculate through the entire system uninterrupted overnight until a stable baseline

was obtained. Oxytocin used for preparation of the standard curve was freshly prepared (1 mg/ml) in mobile phase buffer. A linear standard calibration curve was generated using dilutions of the nonapeptide stock solutions consisting of 500, 250, 100, 50 and 10 ng/ml dissolved in mobile phase buffer. On the basis of peak area, the interpolation of a least-squares regression equation (weighted linear equation $y = 1.297x - 32.726$) yielded a correlation coefficient (r) > 0.995.

3. Results and discussion

3.1. Solid-phase extraction

Prior to quantitation of biologic samples, interfering compounds that do not need to be identified or measured must be removed. By placing emphasis on sample clean-up, analyses can be performed more rapidly, diluted samples can be concentrated and the sensitivity can be increased [12]. The first step in the isolation and purification of oxytocin from biologic samples should employ solid-phase ODS disposable columns which optimize the extraction efficiency while minimizing proteolysis [13]. Several methods have been described for extracting oxytocin from various biologic fluids (including plasma, serum and urine) using octadecylsilyl silica (ODS) solid-phase extraction (SPE) columns [7,10,11]. We successfully adapted the methods described by Padayachi *et al.* [8] and Yasuda *et al.* [4] for extracting oxytocin from cell culture media supernatants. The extraction efficiency for oxytocin with these published methods is reported to be 71–89%. However, SPE recoveries for oxytocin are known to approach 100% [8,4].

3.2. Immunoaffinity column purification

Antibody immunoaffinity columns have recently been shown to be extremely efficient and powerful tools for the purification of certain biologic compounds prior to their quantitation by high-sensitivity HPLC–ED [14,9]. Polyclonal antibody-based immunoaffinity column purification was utilized as the second step in the isolation of oxytocin from biologic samples. Following antibody column preparation, known concentrations of an authentic oxytocin standard were applied, washed and subsequently eluted from the immunoaffinity column to determine the extraction efficiency (% recovery) for the added nonapeptide. The performance of the immunoaffinity column was verified by measuring the percent recovery of the applied nonapeptide using HPLC–ED. The extraction efficiencies for oxytocin over the concentration range of 10 to 500 ng/ml were greater than 96% in all cases (Table 1). Culture media supernatants that were spiked with a known amount of oxytocin (10–500 ng/ml) displayed recoveries that were greater than 95% in all cases (Table 2). The described polyclonal antibody column in combination with HPLC–ED provides a new analytical approach with the specificity, sensitivity and relative simplicity required to measure oxytocin routinely and gives a better result compared with the use of solid-phase extraction techniques alone.

3.3. HPLC using coulometric detection

HPLC with a suitable on-line detection setup provides an alternative method for measuring many different physiologic compounds. The recent introduction of more sensitive dual channel coulometric electrochemical detectors has enabled the detection of the nonapeptide oxytocin

Table 1
Extraction efficiencies of an authentic oxytocin standard following antibody-based immunoaffinity column purification as measured by HPLC–ED

Octapeptide concentration (ng/ml)		Recovery (%)
Before purification	After purification	
500	501.13 ± 6.73[a]	100.2
250	252.03 ± 5.68	100.8
100	104.81 ± 9.35	104.8
50	56.18 ± 7.28	112.4
10	9.69 ± 0.32	96.9

[a] Values following purification are mean ± standard deviation ($n = 3$).

Table 2
Recoveries of an authentic oxytocin standard from spiked supernatant samples following anti-body-based immunoaffinity column purification as measured by HPLC–ED

Octapeptide concentration (ng/ml)		Recovery (%)
Added	Detected	
500	496.12 ± 6.66[a]	99.2
250	249.51 ± 5.62	99.8
100	102.72 ± 9.17	102.7
50	55.61 ± 7.21	111.2
10	9.59 ± 0.32	95.9

[a] Values following purification are mean ± standard deviation ($n = 3$).

to levels that rival a validated RIA. Prior to the present study, the use of more antiquated amperometric and coulometric electrochemical detectors resulted in lower limits of detection, *i.e.* from approximately 1 ng [15] down to 200 pg (200 fmol) on column [16,17]. In the present study, the lower limit of detection (at a signal-to-noise ratio of *ca.* 3) for oxytocin was 40 pg (40 fmol) and the assay was linear in the range of 10–500 ng/ml (coefficient of determination (r^2) = 0.995). Fig. 1 shows a chromatogram of a blank sample supernatant processed through an immunoaffinity column and analyzed by HPLC–ED, and Fig. 2 shows a representative chromatogram from a typical culture media supernatant. The retention time of oxytocin was found to be 9.72 min.

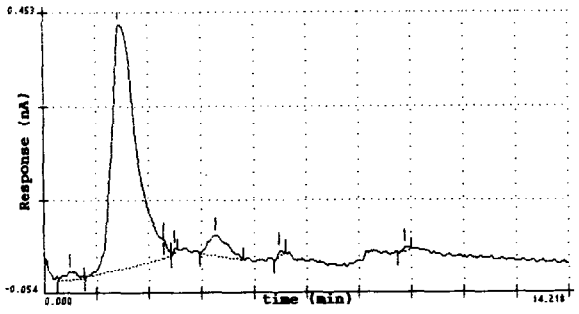

Fig. 1. Chromatogram of a blank culture media supernantant processed through an immunoaffinity column and analyzed by HPLC–ED.

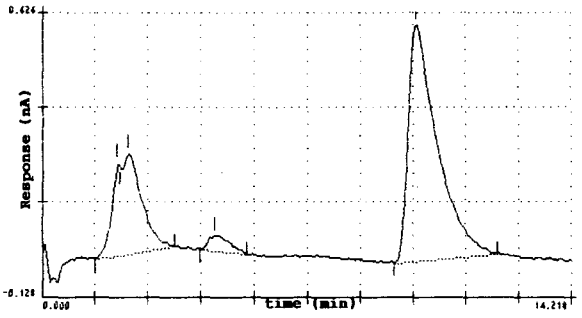

Fig. 2. Chromatogram of a culture media supernatant processed through an immunoaffinity column and analyzed by HPLC–ED containing oxytocin (retention time = 9.72 min).

4. Conclusions

The present study demonstrates that oxytocin can be measured by a straightforward procedure involving solid-phase extraction, immunoaffinity pre-purification and final separation and analysis using HPLC–ED. The dual channel coulometric detection of oxytocin is an extremely attractive alternative to many existing quantitative methods. The procedure described avoids many obvious problems involving complex sample preparation, lengthy gradient elution and pre- or post-column derivatization techniques. This method will undoubtedly prove to be suitable for the identification of related peptides including arginine- and lysine-vasopressin.

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