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Short communication

Extraction of oxytocin and arginine–vasopressin from serum and plasma for radioimmunoassay and surface-enhanced laser desorption–ionization time-of-flight mass spectrometry

David R. Cool*, David DeBrosse

Department of Pharmacology and Toxicology, Wright State University, 3640 Colonel Glenn Hwy., PO Box 927, Dayton, OH 45435, USA

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Abstract

Oxytocin and arginine–vasopressin (AVP) are secreted into the blood in low concentrations. To analyze these peptides, we investigated two common extraction procedures, acetone–ether precipitation and C_{18}-SepPak columns. Recovery from both procedures approached 70–80% of the spiked amount, though the SepPak columns were more efficient. C_{18}-SepPak columns were used to sequentially separate oxytocin from AVP by eluting oxytocin first with 98% acetone followed by elution of AVP with 80% acetonitrile. Surface-enhanced laser desorption–ionization time-of-flight mass spectrometry (SELDI-TOF MS) was used to analyze oxytocin and AVP extracted with C_{18}-SepPak columns from an autistic patient's plasma sample. We conclude that C_{18}-SepPaks provide more consistent and efficient peptide extraction from serum or plasma that augments both quantitative and qualitative analysis by radioimmunoassay and SELDI-TOF MS.

Keywords: Oxytocin; Arginine–vasopressin

1. Introduction

Oxytocin and arginine–vasopressin (AVP) are peptide hormones composed of nine amino acids, derived from the intracellular cleavage of related precursor prohormones and released into the blood to regulate endocrine and neuroendocrine responses throughout the body [1–5]. Due to the small concentrations secreted and their short half-life, peptide hormones, such as AVP and oxytocin, are normally found in the blood in relatively low concentrations (<20 pg/ml) [6–9]. Furthermore, the small sample volumes often obtained from animal experiments (<300 μl of plasma) are not sufficient for multiple analyses of peptides from the same sample and are often irreplaceable. Thus, it would be advantageous to extract and separate peptides of interest from the same sample for independent analysis. While the extraction can be accomplished using acetone–ether precipitation or C_{18}-SepPaks, the separation of the two peptides is made difficult because oxytocin and AVP have nearly identical primary amino acid sequences, i.e. CYFNPCRG for AVP (1084.25 daltons) vs. CYINPCLG for oxytocin (1009.22 daltons). We provide evidence that these two peptides can be separated from each other using C_{18}-SepPaks and a

*Corresponding author. Tel.: +1-937-775-2457; fax: +1-937-775-7221.
E-mail address: david.cool@wright.edu (D.R. Cool).

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multiple solvent system in preparation for quantitative analysis by radioimmunoassay (RIA).

In addition to quantitation of peptide levels, qualitative analyses may also be required for more in depth studies of plasma peptide hormones, e.g. mass. We used surface-enhanced laser desoprtion–ionization time-of-flight mass spectrometry (SELDI-TOF MS) to analyze plasma from an autistic child for the oxytocin peptide. SELDI-TOF MS (Ciphergen, La-Jolla, CA, USA). utilizes chemically treated metal chips that specifically bind peptides and proteins based on their chemical characteristics [10,11]. Following a wash step, peptides and proteins retained on the chips are analyzed by time of flight mass spectrometry. The aim of the present paper was to compare the extraction qualities of two protein extraction methods, C_{18}-SepPaks and acetone–ether, followed by peptide analysis using RIA and SELDI-TOF MS ProteinChip technology.

2. Experimental

2.1. Oxytocin and AVP radioimmunoassay

All chemicals are of reagent grade. Acetone, acetonitrile and trifluoroacetic acid were HPLC grade. Oxytocin (Bachem) and AVP (Bachem) were iodinated by the chloramine T method and separated from free ^{125}I by C_{18}-SepPak (New England Nuclear). [^{125}I]Oxytocin or [^{125}I]AVP was diluted in a buffer containing 1× PBS, 1% BSA and 1 m*M* phenylmethylsulfonylfluoride (PMSF) (RIA buffer). Unlabeled ligand inhibition of binding and displacement curves were generated using oxytocin antibody concentrations (1:30 000) or AVP antibody concentrations (1:25 000) that yielded 20–30% [^{125}I]oxytocin or [^{125}I]AVP binding. Separation of bound and free [^{125}I]oxytocin or [^{125}I]AVP was achieved by addition of 20 μl horse serum followed immediately by 1 ml of 18% polyethylene glycol 8000. After centrifugation and removal of the supernatant, the pellet was counted for 1 min in a MicroMedia 4/200 Plus automatic gamma counter. The resulting cpm were analyzed by a four parameter method [12]. For oxytocin, the average effective dose (ED) ED_{20} was 15±0.13 pg/ml, the average ED_{50} was 3.8±0.4 pg/ml, the average ED_{80} was 0.62±0.25 pg/ml. For AVP, the ED_{20} was 21±7 pg/ml, the ED_{50} was 5.3±1.9 pg/ml and the ED_{80} was 0.74±0.3 pg/ml. The limit of quantitation by RIA for oxytocin and AVP was determined to be 0.49±0.1 and 0.66±0.18 pg/ml, respectively.

2.2. C_{18}-SepPak and Accubond peptide extraction

SepPak (Millipore) C_{18} and Accubond (Fisher) columns were pre-conditioned with 1 ml methanol, followed by 1 ml of distilled water. Various concentrations of oxytocin were added to heat-inactivated horse serum and passed through the columns. The columns were washed with 6 ml of water followed by 3 ml of 3% acetone and fractions collected. The peptides were eluted by 3 ml of 98% acetone followed by 3 ml of 80% acetonitrile containing 0.1% TFA (v/v) and 1 ml fractions were collected. All samples were evaporated to dryness on a Savant Speed Vac and reconstituted in 0.2 ml of RIA buffer.

2.3. Acetone–ether peptide extraction

Ice-cold acetone was mixed with 1 ml of heat-inactivated horse serum containing different concentrations of oxytocin and centrifuged in a Beckman J-6B centrifuge at 3500 rpm for 20 min. The upper, acetone layer was transferred to a clean glass tube and 1 ml of petroleum ether (ether) added, mixed and allowed to sit for 5 min. The upper ether layer was discarded and the lower layer was evaporated to dryness on a Savant Speed Vac. The dried residue was resuspended in 0.2 ml RIA buffer for RIA analysis.

2.4. Ciphergen ProteinChip SELDI-TOF MS

For analyzing plasma oxytocin from an autistic child, 1 ml of plasma was extracted with a C_{18}-SepPak as outlined above and eluted with 80% acetonitrile. The samples were evaporated to dryness and each tube was reconstituted in 25 μl 0.1 *M* HCl. From this, 1 μl was spotted onto a weak cation-exchange (WCX2) protein chip for 30 min in a moist chamber followed by washing with distilled water. Matrix, alpha-cyano-4-hydroxy cinnamic acid

(CHCA) in 50% acetonitrile containing 0.1% TFA (0.5 μl) was added to the spots and allowed to dry. The chip was analyzed with a spot protocol that ionized each spot four times over 20 different areas. The spot was initially ionized one laser hit at 200 intensity, with the sensitivity set at 10, followed by four laser hits at 195 intensity. The source voltage was set at 20 000 V and detector voltage at 1900 V. The 80 laser hits were averaged for each spot and the resulting protein profile analyzed to determine the mass of each peak. An aliquot of the eluate from the autistic patient sample was analyzed by RIA for oxytocin. Oxytocin and AVP standards (20 pg/μl) were also analyzed by SELDI-TOF MS in serum, were spotted on WCX2 chips and analyzed with the same spot protocol used for analysis of the plasma from the autistic child.

3. Results and discussion

3.1. Comparison of acetone–ether with C_{18}-SepPak and Accubond columns

The acetone–ether procedure was used to extract [^{125}I]oxytocin or [^{125}I]AVP from serum (1 ml each). Addition of acetone to serum causes precipitation of larger proteins while smaller peptides remain in the aqueous phase. Approximately 15% of the [^{125}I]oxytocin and 13% of the [^{125}I]AVP cpm were precipitated following addition of the acetone to the spiked serum samples (Fig. 1A). The aqueous layer was transferred to another tube, ether added and mixed. After 5 min, the upper ether layer was removed to a clean tube. Less than 8% of the [^{125}I]oxytocin and 12% of the [^{125}I]AVP were removed with the ether layer. The aqueous layer contained the majority of the [^{125}I]oxytocin or [^{125}I]AVP cpm (~75–77% for both).

C_{18}-SepPaks were loaded with an aliquot of the same serum (1 ml) containing [^{125}I]oxytocin or [^{125}I]AVP. Less than 3% of the [^{125}I]oxytocin or 7% of the [^{125}I]AVP cpm were observed in the flow through and water wash fractions, indicating a high efficiency of retention on the column (Fig. 1B). In addition, less than 4% of the [^{125}I]oxytocin cpm were found in the 3% acetone wash fraction. Approximately 92% of the cpm were eluted with 98% acetone (Fig. 1B). Less than 1% remained bound to the SepPak columns. Accubond C_{18} columns yielded a profile, in which ~40% of the cpm ([^{125}I]oxytocin or [^{125}I]AVP) were found in the flow through (Fig. 1C). The results suggest that less [^{125}I]oxytocin or [^{125}I]AVP was lost during the initial wash steps from the C_{18}-SepPaks than from the Accubond columns or in the precipitation with acetone–ether.

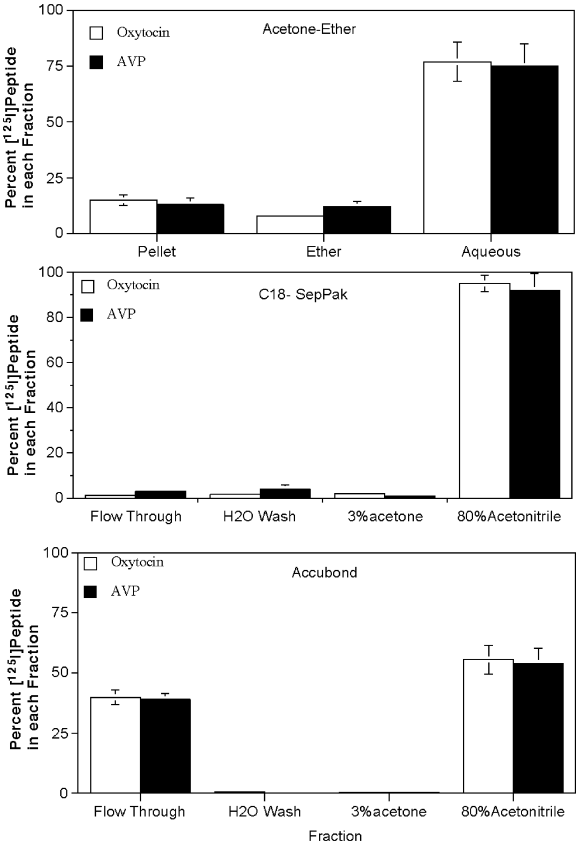

Fig. 1. Relative amount of [^{125}I]oxytocin ([^{125}I]OT) or [^{125}I]AVP recovered using acetone–ether, C_{18}-SepPak or Accubond Column extraction procedures. The cpm added were ~5000–6000 per assay. (A) Pellet, represents the percent of [^{125}I]oxytocin or [^{125}I]AVP remaining in the pellet after addition of acetone. Ether represents the percent cpm removed from the aqueous phase which contains the majority of the iodinated peptides. (B) Extraction of [^{125}I]oxytocin or [^{125}I]AVP using C_{18}-SepPak columns. (C) Extraction of [^{125}I]oxytocin or [^{125}I]AVP using Accubond columns. H_2O wash indicates washing the column with distilled water. The data represent the mean±SEM of duplicate measurements from three experiments.

3.2. Comparison of acetone–ether with SepPak and Accubond columns

Preliminary studies using iodinated oxytocin and iodinated AVP suggested that a higher recovery could be obtained using the SepPak extraction procedure. The extraction efficiency of acetone–ether, SepPaks and Accubond columns was compared by extracting serum (1 ml) containing; 0, 1, 2.5, 5, 10 and 20 pg of unlabeled oxytocin. The amount recovered for each concentration was determined by RIA. In Table 1, acetone–ether extraction gave values higher than the absolute amount spiked through 2.5 pg oxytocin. From 5 to 20 pg, the recovery decreased from 78 to 62% of the spiked values, respectively. The percent recovery from C_{18}-SepPak columns ranged between 65% at 1.25 pg/ml to a high of 80% at 10 pg/ml spiked oxytocin (Table 1). Accubond columns were less efficient, yielding only 21–54% recovery at each concentration (Table 1). In addition, a salt crystal was often found in the tube after evaporating the aqueous phase to dryness. When the sample with the salt was resuspended for RIA analysis, the increased salt concentration was found to affect the RIA. Using the standard error of the mean (SEM) as an indicator of the variability in the three assays; the C_{18}-SepPak columns provided the least variability among the three methods. The percent recovery using the C_{18}-SepPaks was consistently higher than acetone–ether precipitation. Together, these results suggest that the C_{18}-SepPaks are more efficient and more accurate than acetone–ether precipitation or accubond columns and are the preferred method for extracting oxytocin from serum or plasma samples.

3.3. Extraction and separation of oxytocin and AVP using C_{18}-SepPaks

Oxytocin and AVP, 20 pg of each, were mixed with serum (1 ml) and loaded onto a single C_{18}-SepPak column. The column was washed with H_2O and 3% acetone. The column was eluted with 98% acetone followed by elution with 80% acetonitrile. Fractions were collected, evaporated to dryness and reconstituted in 200 μl of RIA buffer and analyzed by RIA. Water and 3% acetone washes caused a negligible release of either peptide (<15%). Elution with 98% acetone resulted in release of 80% of the oxytocin and 15% of the AVP (Fig. 2A and B, respectively). Elution with 80% acetonitrile caused the remainder of the oxytocin to be released from the SepPak as well as AVP (>80%) (Fig. 2A,B). These results are similar to those reported previously [13]. Since acetone–ether precipitation is not capable of separating the oxytocin and AVP peptides, SepPak extraction is the better method for extracting oxytocin and AVP from the same sample for separate analysis. Microliter pipet tips containing C_{18}-SepPak material have been found to provide similar separations, though in much smaller volumes, i.e. <50 μl.

3.4. Qualitative SELDI-TOF MS analysis of oxytocin and AVP

SELDI-TOF MS is a relatively new mass spectrometric technique in which proteins and peptides are applied to chemically treated metal chips, the chips washed with a buffer and the proteins that are retained on the chips analyzed by time-of-flight mass

Table 1
Percent recovery of oxytocin using acetone–ether, C_{18}-SepPaks or Accubond columns

Method	Oxytocin spiked (pg/ml)[a]				
	1.25	2.5	5.0	10.0	20.0
Acetone–ether	143±29[b]	110±11	79±7	62±4	61±8
C_{18}-SepPak	65±5	73±3	73±3	80±3	76±7
Accubond	54±22	21±14	28±12	24±3	45±7

[a] Increasing concentration of oxytocin were added to 1 ml of serum and extracted using actone–ether, C_{18}-SepPak columns and Accubond columns. The amount of oxytocin recovered was determined by RIA.

[b] Results represent the average percent recovered±standard error of the mean (SEM) for three separate experiments.

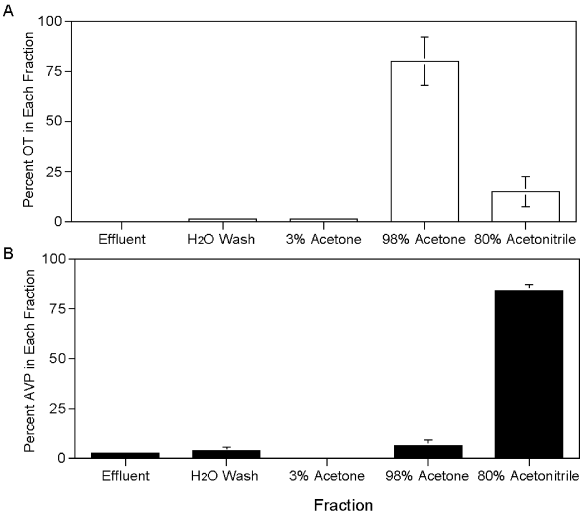

Fig. 2. Extraction and separation of oxytocin (A) and AVP (B) using C_{18}-SepPaks. The SepPak column was loaded with 20 pg of oxytocin and AVP in 1 ml of serum. The columns were washed with 6 ml of distilled water followed by 3 ml of 3% acetone. The peptides were eluted from the SepPak with 3 ml of 98% acetone and with 3 ml 80% acetonitrile. Fractions were collected and the amount of peptide determined by RIA. The results represent the mean±SEM for three experiments.

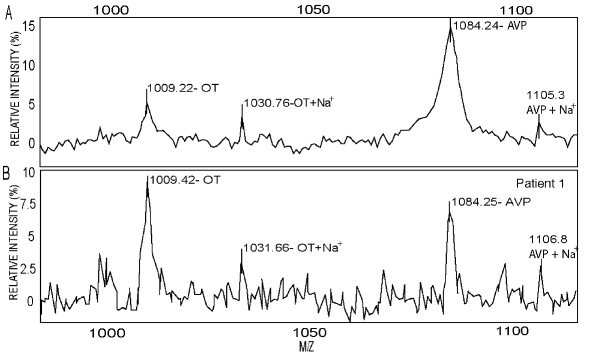

Fig. 3. SELDI-TOF MS ProteinChip analysis of oxytocin and AVP. (A) Serum containing 20 pg/μl of oxytocin (OT) and AVP was analyzed by SELDI-TOF MS on a WCX2 proteinchip. The spectrum revealed peaks for oxytocin (1009.22 M_r), oxytocin+sodium (1030.56 M_r), AVP (1084.21 M_r) and AVP+sodium (1105.56 M_r). These peaks were absent when oxytocin and AVP were omitted from the serum (data not shown). (B) Plasma from the blood of a child diagnosed with autism was extracted by C_{18}-SepPak and analyzed by SELDI-TOF MS. The peaks for OT (1009.22 M_r), OT+sodium (1031.66 M_r) AVP (1084.25 M_r) and AVP+sodium (1106.80 M_r) were observed.

spectrometry. Oxytocin and AVP standards (20 pg each) were analyzed on WCX2 chips (weak cation-exchange) using SELDI-TOF MS (Fig. 3A). The mass determined for each peptide following SELDI-TOF MS was 1009.22 M_r for oxytocin and 1030.76 M_r for its sodium peak. Likewise, AVP was seen as a single peak of 1084.24 M_r with its sodium peak at 1105.3 M_r. Limitations to SELDI-TOF MS are similar to comparable MALDI-TOF MS systems, i.e. low concentration of peptides, high salt concentrations, detergents and other contaminants that can cause low sensitivity and even suppression of the peptide peak intensity [14–16]. Therefore, prior to attempting SELDI-TOF MS, it was first necessary to extract the peptides from the plasma using C_{18}-SepPaks.

Plasma (1 ml) from the blood of a 7-year-old male diagnosed with autism was extracted and concentrated using a C_{18}-SepPak that was washed and eluted with 80% acetonitrile. When the eluate was analyzed by SELDI-TOF MS using a WCX2 chip, peaks corresponding to oxytocin, AVP and their corresponding sodium ions, were identified (Fig. 3B, patient sample). The observation of oxytocin at 1009.42 M_r and its sodium adduct at 1031.66 M_r suggests that in this patient, the correct processing of oxytocin was not altered as had been proposed by other researchers [8,17].

The concentration of oxytocin in the autistic patient sample was determined by RIA to be 4.16 pg/ml. This value is higher than previously published values that were obtained using the acetone–ether method for extracting patient plasma [8], but consistent with other studies on plasma oxytocin levels [6,7].

4. Conclusions

The methods outlined in this paper show that C_{18}-SepPak columns provide better efficiency of recovery and accuracy for peptide hormone extraction from serum than acetone–ether precipitation or Accubond columns. This paper is the first to describe the analysis of the peptide hormones, oxytocin and

AVP, in blood samples using SELDI-TOF mass spectrometry.

5. Nomenclature

AVP	arginine–vasopressin
OT	oxytocin
SELDI-TOF MS	surface-enhanced laser desorption–ionization time-of-flight mass spectrometry
RIA	radioimmunoassay
ELISA	enzyme-linked immunosorbant assay

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