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Evaluation of enzyme immunoassay and radioimmunoassay methods for the measurement of plasma oxytocin

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Abstract

Objective—There is increased interest in measuring peripheral oxytocin levels to better understand the role of this peptide in mammalian behavior, physiology, and disease. The purpose of this study was to compare methods for plasma oxytocin measurement using a commercially available enzyme immunoassay (EIA) and radioimmunoassay (RIA), to evaluate the need for sample extraction, and to assess the immunospecificity of the assays.

Methods—Oxytocin was measured in extracted and unextracted human plasma samples (n = 39). Oxytocin and its degradation products were separated by high performance liquid chromatography and gel filtration chromatography and then assayed by EIA or RIA to identify oxytocin immunoreactive peaks.

Results—Without extraction, plasma measured by EIA was more than 100-fold higher than in extracted plasma, and the correlation between oxytocin levels in extracted and unextracted plasma was minimal (Spearman's rho = −0.10, p = 0.54). Using the RIA, most samples (> 90%) were below the level of detection with or without extraction. Following chromatographic fractionation of sample extracts, multiple immunoreactive products were found to be present in addition to oxytocin, which casts doubts on the specificity of the assays.

Conclusions—Changes in oxytocin levels have been reported in social and behavioral challenge studies. This study indicates that sample extraction is necessary to obtain valid assay results. Changes in oxytocin degradation products are likely to contribute to the previously observed responses in circulating oxytocin levels to behavioral and social challenge. There is a critical need for validated and reliable methods to measure oxytocin in biological samples.

Keywords

Oxytocin; Extraction; Immunoassays

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Introduction

The neurohypophyseal peptide oxytocin, which acts in the central nervous systems of males and females, plays an important role in a variety of complex social behaviors including affiliation, sexual behavior, social recognition, stress buffering, aggression, and trust (1-9). There has been increased interest in quantifying peripheral oxytocin levels to better understand oxytocin's role in human and animal social behavior. Oxytocin has been measured by immunoassays in biological fluids including plasma, saliva, urine, and cerebrospinal fluid in different mammalian species. Sample preparation often involves extraction (10-13), but several reports have utilized direct measurement (9, 14). Reported plasma oxytocin levels vary from 1 to 300 pg/ml in human and mammalian species, however, significant differences exist between values observed in similar populations and are likely due to methodological differences, making comparison between studies difficult (Table 1). Lower oxytocin values have usually been obtained from samples subjected to extraction, while higher values occurred in samples assayed directly, suggesting that sample preparation influences quantification. The purpose of this study was to compare two commercially available methods1 for oxytocin measurement using enzyme immunoassay (EIA) or radioimmunoassay (RIA), to determine the stability of plasma oxytocin, to evaluate the need for sample extraction, and to assess the immunospecificity of the assays.

Methods

Oxytocin Assays

Oxytocin EIA (Assay Designs, Ann Arbor, MI) and RIA (Phoenix Pharmaceuticals, Burlingame, CA) were performed following the manufacturers' protocols. Limits of detection were 1.2 pg/well and 1.0 pg/tube. These values would equate to 0.12 and 0.1 pg/ml for unextracted samples or 1.2 and 1.0 pg/ml after extraction of 1 ml plasma. Intra- and inter-assay variability were 9% and 15%, 7% and 15%, for EIA and RIA, respectively, as reported by the manufacturers. Unextracted samples measured by EIA were diluted 1:4 as described by Kramer et al. (14).

Sample Extraction

Sample extraction is a procedure to eliminate the effect of potentially interfering molecules, reduce sample matrix effects, and concentrate and enrich the analyte of interest from the sample prior to analysis. Sample extraction was evaluated using two different methods: solid phase extraction (the method recommended by both oxytocin assay manufacturers), and solvent extraction (a method previously validated for the measurement of oxytocin (10). De-identified human EDTA plasma or serum samples were provided by the University of Miami Diabetes Research Institute Clinical Chemistry Laboratory and stored at −80°C until assay. Solid phase extraction of samples was performed using 200 mg C18 Sep-Pak columns (Bachem, San Carlos, CA). Columns were equilibrated with 3 ml of acetonitrile, then twice with 3 ml of 0.1% trifluoroacetic acid (TFA). Up to 1 ml of plasma was mixed with an equal volume of 0.1% TFA, centrifuged at 14,000 g for 20 minutes at 4°C, and the acidified and clarified plasma was then applied to the column. The flow-through fraction was discarded, columns were washed once with 3 ml of 0.1% TFA, then twice with 3 ml of water. Oxytocin was eluted with 3 ml of 60% acetonitrile. The solvent was evaporated under a stream of nitrogen gas, and the sample completely dried by lyophilization. In order to evaluate different extraction procedures, some samples were extracted using an organic solvent

[1]At the time the study was conducted, the two oxytocin assay methods evaluated were the only commercially available kits on the market. Since that time, the manufacturer of the RIA kit (Phoenix Pharmaceuticals) has marketed two ELISA format kits that were not evaluated in this study.

method as described previously (10). Briefly, 1 ml plasma was mixed with 2 ml of ice-cold acetone, centrifuged at 1,200 g for 20 minutes at 4°C, the supernatant was transferred, and washed with 2 ml anhydrous ether. The extract was dried under a stream of nitrogen gas. For immunoassay, samples were reconstituted in 0.12 ml of assay buffer provided by the EIA or RIA kits. For HPLC fractionation (see below), 4 or 10 ml of plasma were extracted (1 ml/column) and the eluates pooled to provide sufficient levels of oxytocin for detection by EIA or RIA, respectively.

Extraction efficiency was determined by spiking plasma with approximately 20,000 cpm/ml of ^{3}H-oxytocin (Perkin Elmer, Waltham, MA) and fractions (i.e., flow-through, washes, eluate) were collected and ^{3}H determined by scintillation counting.

Chromatography

Chromatography is a procedure that separates molecules based on their physical properties. High performance liquid chromatography (HPLC) separates molecules based on hyrodphobicity, such that polar compounds are eluted first. Gel filtration chromatography separates molecules based upon their molecular weight by utilizing a porous gel.

Oxytocin and its degradation products were separated by high performance liquid chromatography (HPLC) on a Phenomenex C18 (4.6×150 mm) column using a Hitachi LaChrom Elite HPLC system (Schaumburg, IL). Samples were eluted at a flow rate of 1 ml per min with 20% (v/v) acetonitrile/0.1% TFA for 9 minutes, then linearly increased to 100% acetonitrile/0.1% TFA over four minutes, and maintained in this buffer until the end of the run. Chromatography was monitored by ultraviolet absorbance at 280 nm. Plasma extracts (100 μl) were injected into the column and 0.75 ml fractions were collected.

Gel filtration chromatography was performed using a Superdex 75 10/300 GL column (GE Healthcare, Piscataway, NJ) eluted with ammonium acetate buffer (5 mM; pH = 7.8) at a flow rate of 1.0 ml per min and monitored at 280 nm. Plasma (400 μl) or plasma extracts (200 μl representing 4 ml of plasma) were injected into the column and 1.5 ml fractions were collected.

After chromatography, samples were lyophilized reconstituted with 0.12 ml assay buffer and assayed by EIA or RIA to identify oxytocin immunoreactive peaks.

Statistical analyses

Data were presented as mean ± standard error of the mean (SEM). Oxytocin values that were below the level of detection were assigned a value of 0.1 pg/ml for statistical analysis. Results were compared by paired t-tests between groups and relationships between variables were examined by Pearson product-moment and Spearman rank correlation coefficients using SPSS for Windows (Version 17, Chicago, IL). An alpha level of 0.05 was required for statistical significance.

Results

Extraction and Stability of Oxytocin

Two sample extraction methods were evaluated based on the recovery of added ^{3}H-oxytocin to the sample. First, solid phase extraction on a C-18 column resulted in an extraction efficiency for plasma (n=6) and serum (n=3) samples of 92% ± 13% and 81% ± 5%, respectively. Second, using the organic solvent extraction method recovery of oxytocin in plasma (n=4) was 72% ± 2%. Given the higher recovery in plasma using the solid phase extraction, this method was used in subsequent experiments.

Initial studies assessed the stability of oxytocin in plasma. ^{3}H-oxytocin was added to the samples, incubated at 0°, 22°, or 37°C for 2 or 18 hours, or subjected to several freeze/thaw cycles. The samples were then extracted and intact ^{3}H-oxytocin was determined by HPLC. Extraction efficiency was similar for samples under the various conditions (95 ± 12%). HPLC analysis revealed that greater than 95% of the extracted ^{3}H-oxytocin remained intact at all temperatures and incubation times (data not shown). After five freeze/thaw cycles, 87% of the oxytocin remained intact. These data indicate that these temperature conditions and extraction procedures had minimal effects on oxytocin stability.

Measurement of plasma oxytocin by EIA and RIA in extracted and unextracted samples

Oxytocin levels were compared between extracted and unextracted plasma from the same samples (n = 39) using EIA and RIA. For EIA, mean values were 1.8 ± 0.4 pg/ml in extracted samples and 358 ± 70 pg/ml in unextracted samples ($p < 0.01$ between groups). Oxytocin values measured in extracted samples did not correlate with values in unextracted samples by EIA using Pearson's product moment correlation ($r = 0.09$, $p = 0.60$; Figure 1). Log transformation of the data did not result in a significant correlation ($r = -0.14$, $p = 0.40$). Given the non-normal distribution of values generated for extracted and unextracted samples, the data were also analyzed using nonparametric methods (Spearman rank correlation), which also revealed no significant association ($r = -0.10$, $p = 0.54$). By RIA, plasma oxytocin levels in 22 of 25 extracted samples and 19 of 25 unextracted samples had values below the limit of detection (<1.0 pg/tube). Thus, the RIA lacked sufficient sensitivity in more than 90% of samples to measure plasma oxytocin levels even after extraction and the resulting 10-fold concentration of the sample.

Spike-and-recovery experiments were performed to determine whether analyte detection is affected by the biological sample matrix (in this case plasma) and analytical recovery in samples not extracted and to evaluate the effects of sample extraction on analytical recovery. To evaluate the accuracy of the measurement and to obtain concentrations measurable by both immunoassays, plasma samples were spiked with 50, 25 and 12.5 pg/ml oxytocin prior to assay with and without sample extraction. By EIA, spiking resulted in a linear increase in oxytocin concentrations ($y = 1.16x - 2.51$, $r = 0.98$, $p < 0.02$, where y is the measured oxytocin value in and x is the expected oxytocin value based on the added spike and recovery of the spike was 97 ± 6% in extracted samples. In unextracted, diluted plasma, spiking the samples resulted in a linear increase in oxytocin concentrations ($y = 17.9x + 218$, $r = 0.98$, $p < 0.02$), but in contrast to extracted samples, recovery of the spike was 364 ± 108%. Using RIA, spiking resulted in a linear increase in plasma oxytocin concentrations after solid phase extraction ($y = 1.2x - 6.2$, $r = 0.99$, $p < 0.01$), and recovery of the immunoreactivity was 85 ± 10%. In unextracted samples, there was no linear increase in oxytocin concentrations ($y = -0.15x + 22.7$, $r = 0.33$, $p = 0.70$) and recovery of the spike was 18.8 ± 9%. In spiked extracted samples that provided values in both EIA and RIA assays, results were highly correlated ($r = 0.98$, $p < 0.01$). These data demonstrate the linearity and accuracy of the measurements in extracted samples by both methods. Unextracted samples resulted in over-recovery of spiked oxytocin by EIA and under-recovery by RIA indicative of sample matrix effects affecting the measurement.

Fractionation of samples and characterization of immunoreactive products

As mentioned, observed oxytocin values were orders of magnitude higher in unextracted samples as opposed to extracted samples, which suggested the presence of non-oxytocin immunoreactive species that were removed by extraction. In order to evaluate the immunospecificity of the EIA, samples were subjected to chromatographic fractionation and immunoassay of the separated fractions. Using gel filtration chromatography, which separates molecules based upon size, at least three distinct peaks of immunoreactivity were

observed in unextracted plasma (Figure 2A). Only the third peak eluted in the region that coincided with authentic oxytocin (MW=1009), and this peak accounted for approximately 55% of the total immunoreactivity. The same plasma was subjected to solid phase extraction followed by the same chromatography and immunoassay. After extraction, only a single peak of immunoreactivity, coincident with the elution of authentic oxytocin, was detected (Figure 2B). It is worth noting that this peak accounted for 20 to 40% of the immunoreactivity seen in the third peak of the unextracted sample, suggesting that extraction removed over 60% of the immunoreactivity in this “oxytocin peak”. These data demonstrate that immunoreactivity with molecular mass greater than that of oxytocin is present in plasma and contributes to the high values reported in unextracted plasma samples.

To further evaluate the nature of the immunoreactive species present in the extracted samples, plasma extracts were subjected to reverse phase HPLC, which separates molecules based on their hydrophobicity, and the collected fractions tested for oxytocin by EIA (Figure 3). In three different plasma samples subjected to HPLC fractionation, two or three distinct peaks of oxytocin immunoreactivity were detected by EIA. In all cases, the peak that co-eluted with the position of authentic oxytocin was not the major peak of immunoreactivity. Oxytocin, based on elution position, accounted for only 25%, 15%, and 7% of the total immunoreactivity (Figure 3A, 3B, and 3C, respectively). Samples subjected to extraction with organic solvent yielded HPLC profiles with similar multiple immunoreactive peaks (data not shown). In two other samples analyzed by RIA following HPLC separation, multiple peaks of oxytocin immunity reactivity were again observed, and only 22% or 43% of the total immunoreactivity co-eluted with authentic oxytocin (Figure 3D, 3E, respectively).

Discussion

The results of the current study raise serious concerns regarding the use of commercially available assays for the measurement of plasma oxytocin. Assay results following chromatographic fractionation of unextracted plasma show the presence of multiple immunoreactive species. Extraction resulted in removal of larger molecular weight species that accounted for about half of the total immunoreactivity, as demonstrated by the gel filtration profile. In addition, oxytocin immunoreactivity of fractions eluting with the molecular size of oxytocin was reduced by 57-78% after extraction. While the reduction of oxytocin immunoreactivity from this peak varied between samples, these data suggest that there are also small molecular weight substances in plasma that contribute to the oxytocin immunoreactivity and are removed by extraction. Additional fractionation of extracted samples by HPLC, which separates molecules based on hydrophobicity and not size, clearly showed that the majority of immunoreactivity did not coincide with authentic oxytocin, and in the worst case the non-oxytocin peaks represented greater than 93% of the total immunoreactivity.

Preliminary studies from our laboratory examining oxytocin degradation suggest that at least one of the immunoreactive peaks identified by HPLC is consistent with an oxytocinase degradation product (unpublished observation). Results from several studies have shown that social and behavioral manipulations result in changes in plasma oxytocin immunoreactivity using the methods evaluated in this study and in the absence of sample extraction. It is possible that what is being measured is both plasma oxytocin and oxytocin degradation products, and that the degradation products contribute to the measured changes in circulating oxytocin levels. Given the short half-life of plasma oxytocin (3 to 6 minutes, (15)) it may be reasonable to assume that degradation products are more stable than the parent oxytocin, may accumulate in plasma, and are amenable to measurement. Clearly, the

identity of the immunoreactive molecules and the specificity of the antibodies used in the immunoassays need to be elucidated if these assumptions are to be supported.

The present study suggests there are several other issues important in the measurement of plasma oxytocin. Two commercially available assays for plasma oxytocin were compared and it was found that sample extraction (strongly recommended by both manufacturers) is a critical variable for accurate measurement. Without extraction, plasma oxytocin levels by EIA were more than 100-fold higher compared to the same extracted sample, and there was no correlation between oxytocin levels in extracted and unextracted plasma. The effect of extraction was also evident in samples measured by RIA where unextracted plasma gave values that were at least 10-fold higher than the extracted sample.

The commercially available RIA lacks sensitivity to measure basal plasma oxytocin, resulting in values below the level of detection with or without extraction (<10% samples had detectable levels). In a recent study from our laboratory (13) using extracted human plasma samples, 49% of females and 67% of males had oxytocin values below the limit of detection by RIA. In another study that used the same RIA, greater than 6 ml of plasma had to be extracted in order to improve detection limits, which resulted in measured oxytocin levels between 0.1 and 4.6 pg/ml (16). In contrast, the present study showed less than 5% of the samples measured by EIA had undetectable levels.

In summary, the current study compared two commercially available immunoassays for the quantification of plasma oxytocin levels. Our data showed the importance of sample extraction for these measurements, and assessed the relative sensitivity between the assays. Importantly, further analysis showed the presence of multiple immunoreactive products in addition to oxytocin in sample extracts, which casts doubts on the specificity of the assays. Although the current study only examined oxytocin immunoreactivity in plasma samples, several published reports have assessed oxytocin in other biological fluids (e.g., saliva (17, 18), urine (19, 20)), and the validity of these methods has also been challenged (21-23). Given the increased interest and important role of this peptide in mammalian behavior, physiology and disease, there is a critical need to establish valid and reliable methods to measure oxytocin in plasma and other biological fluids.

Acknowledgments

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Abbreviations

EIA	enzyme immunoassay
RIA	radioimmunoassay
HPLC	high performance liquid chromatography
SEM	standard error of the mean
MW	molecular weight

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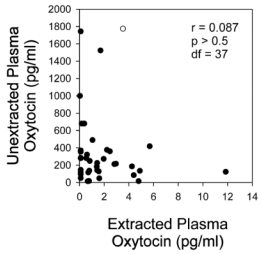

Figure 1.

Comparison of oxytocin levels in plasma (n = 39) with and without extraction. One milliliter of plasma was extracted using 200 mg C18 Sep-Pak columns as described in the Methods. Oxytocin levels in plasma (diluted 1:4 before assay) and the corresponding extract were assayed by the EIA method, and the results are expressed as pg/mL of the original plasma volume. Five of 39 samples had values below the limit of detection and were assigned a value of 0.1 pg/mL for the statistical analysis. Correlation was determined using the Pearson correlation coefficient.

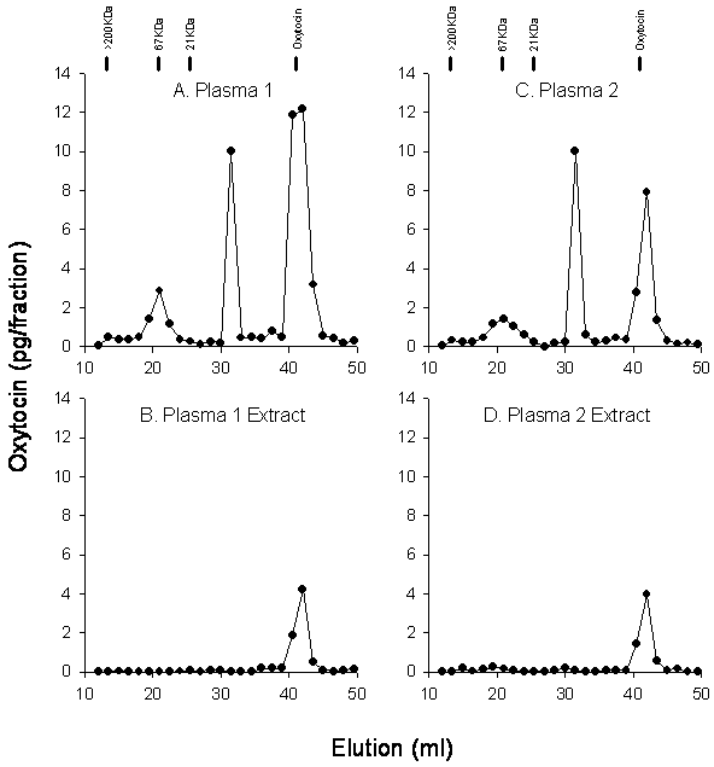

Figure 2.

Oxytocin immunoreactivity in plasma and plasma extracts after gel filtration chromatography. Plasma (400 μL, A and C) or extracts of the same samples (B and D) were applied to a Superdex 75 10/300 GL column and eluted with 5-mM ammonium acetate buffer (pH 7.8) at a flow rate of 1.0 mL/min, and 1.5-mL fractions were collected. After collection, the fractions were lyophilized then reconstituted and assayed for oxytocin immunoreactivity by EIA. Samples 1 and 2 had plasma oxytocin levels of 442 and 281 pg/mL, respectively, and after extraction, 15.2 and 6.1 pg/mL, respectively. Elution of MW markers and of oxytocin is indicated above A and C.

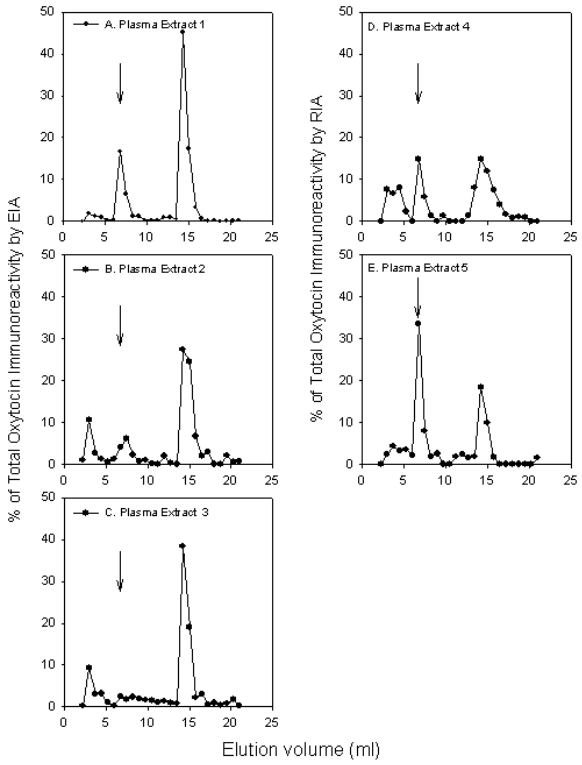

Figure 3.

Oxytocin immunoreactivity profile in plasma extracts after high-pressure liquid chromatography fractionation. Plasma extracts of different samples were performed as described in the Methods. After collection, the fractions were lyophilized then reconstituted and assayed for oxytocin immunoreactivity by EIA (A–C) or RIA (D and E). Samples 1, 2, and 3 had plasma oxytocin levels of 15.5, 10.1, and 3.4 pg/mL, respectively. Samples 4 and 5 had plasma oxytocin levels of 13.3 and 4.1 pg/mL, respectively. Arrows indicate elution of the oxytocin standard.

Table 1

Reported Oxytocin Levels Measured Using Different Methods

Species	Sex	Sample	State	Concentration (pg/ml)	Measurement	Extraction	Reference
Humans	F	Plasma	Baseline	23 ± 11.2	EIA	Y	(24)
Humans	F/M	Plasma Saliva	Control	7.5 ± 1.4 6.5 ± 1.8	EIA	Y	(18)
Humans	F	Plasma Saliva	Baseline	4.8 ± 0.5 4.7 ± 0.5	EIA	Y	(11)
Humans	F/M	Saliva	Control	31 ± 12.7 24.4 ± 32	EIA	N Y	(21)
Humans	F/M	Plasma	Control	198 ± 165	EIA	N	(9)
Humans	M	Plasma	Baseline	99 – 421	EIA	N	(25)
Humans	F/M	Plasma	Control	240 ± 224	EIA	N	(26)
Humans	F/M	Plasma	Baseline	259 ± 38	EIA	N	(27)
Humans	F	Saliva		6.44 - 61.05	EIA	N	(28)
Humans	M	Plasma Saliva	Baseline	405 ± 152 7.1 ± 4.0	EIA	N	(17)
Humans	F M	Plasma	Baseline	249 ± 180 273 ± 234	EIA	N	(29)
Rhesus Monkey	M	Plasma	Nurse-reared Mother-reared	225 275	EIA	N	(30)
Prairie voles Rats	F M F M	Plasma	Baseline	488 ± 88 264 ± 31 187 ± 53 79 ± 6	EIA	N	(14)
Rats	M	Plasma	0800	450 – 700	EIA	N	(31)
Rats	M	Plasma	Control	200	EIA	N	(32)
Rats Mice	F	Plasma	Control	60 120	EIA	N	(33)
Mice	F/M	Plasma	CD38−/−	200	EIA	N	(34)
Mice	F/M	Plasma	Control	100 – 120	EIA	N	(35)
Humans	M/F	Plasma	Baseline	4.0	RIA	Y	(10)
Humans	M	Plasma	Control Autistic	1.2 ± 0.8 0.6 ± 0.6	RIA	Y	(36)

Species	Sex	Sample	State	Concentration (pg/ml)	Measurement	Extraction	Reference
Humans	M	Plasma	Normal	0.17	RIA	Y	(37)
Humans	M	Plasma	Control Autistic	1.4 ± .10 0.8 ± .09	RIA	Y	(38)
Humans	F	Plasma	Baseline	3.5 ± 3.5	RIA	Y	(39)
Humans	M	Plasma	Control	2-4	RIA	Y	(40)
Humans	M	Plasma	Baseline	71	RIA	Y	(41)
Humans	F M	Plasma	Baseline	1.7 ± 0.2 1.5 ± 0.2	RIA	Y	(42)
Humans	F	Plasma	Control Birth Control	1.5 - 2.6 2	RIA	Y	(12)
Humans	F/M	Plasma	Control Autistic	5 – 8 8 – 12	RIA	Y	(43)
Humans	F/M	Plasma	Control	0.1 – 4.6	RIA	Y	(16)
Humans	F/M	Plasma	Baseline	2.17 ± 0.4	RIA	Y	(44)
Humans	F	Plasma	Baseline	50 ± 9.6	RIA	Y	(45)
Humans	F	Plasma	Control	1 – 3	RIA	Y	(46)
Humans	F	Plasma	Baseline	5.7 ± 8.1	RIA	Y	(47)
Humans	F/M	Plasma	Control	1	RIA	Y	(48)
Humans	F	Plasma	Baseline	1.6 ± 2.8	RIA	Y	(13)
Cynomolgus Monkey	F	Plasma	Prepartum Parturition	5 22 ± 7	RIA	Y	(49)
Heifers	F	Plasma	Pregnant Baseline	1 - 10	RIA	Y	(50)
Rabbit	M	Plasma	Control	8.8 ± 2.1	RIA	Y	(51)
Rats	M	Plasma Urine	Control	17 ± 22 1.4 ± 0.1	RIA	Y	(52)
Rats	M	Plasma	0800-0900 1700-1800	3.0 ± 0.6 6.6 ± 1.0	RIA	Y	(53)
Rats	M	Plasma	Control	1 – 2	RIA	Y	(54)
Rats	F	Plasma	Control	30.2 ± 8.5	RIA	Y	(55)
Mice	M	Plasma Urine	Baseline OT +/+	50 250.2 ± 35.3	RIA	Y	(20)
Rabbit	F	Plasma	During Birth & Parturition	12 - 3030	RIA	N	(56)

Species	Sex	Sample	State	Concentration (pg/ml)	Measurement	Extraction	Reference
Humans	F/M	Urine	Baseline	14-17 μg/mg creatinine	HPLC	Y	(19)