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## Neurophysin I is an Analytically Robust Surrogate Biomarker for Oxytocin

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### Abstract

Oxytocin is a pleiotropic neuropeptide that plays roles in biological processes ranging from birth, lactation, and social bonding to immune function, cardiovascular repair, and regulation of appetite. Although measurements of endogenous oxytocin concentrations have been performed

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for more than 50 years, the ability to measure oxytocin accurately poses notable challenges. One potential solution for overcoming these challenges involves measurement of oxytocin's carrier molecule – neurophysin I (NP-1) – as a surrogate biomarker. NP-1 is secreted in equimolar concentrations with oxytocin but has a longer half-life, circulates in higher concentrations, and can be measured using a sandwich immunoassay. We report experiments that 1) analytically validate a commercially available NP-1 sandwich immunoassay for use with human plasma and urine samples, 2) confirm the specificity of this assay, based on detection of NP-1 in plasma from wild-type but not oxytocin knockout mice, 3) demonstrate that NP-1 concentrations are markedly elevated in late pregnancy, consistent with studies showing substantial increases in plasma oxytocin throughout gestation, and 4) establish strong correlation between NP-1 and plasma oxytocin concentrations when oxytocin is measured in extracted (but not non-extracted) plasma. The NP-1 assay used in this study has strong analytical properties, does not require time-intensive extraction protocols, and the assay itself can be completed in < 2 hours (compared to 16-24 hours for a competitive oxytocin immunoassay). Our findings suggest that much like copeptin has become a useful surrogate biomarker in studies of vasopressin, plasma measurements of NP-1 have similar potential to advance oxytocin research.

### Keywords

Oxytocin; neurophysin I; immunoassay; measurement; validation; Biomarker

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Oxytocin is a pleiotropic neuropeptide that plays roles in biological processes ranging from birth, lactation, and social bonding to immune function, cardiovascular repair, and regulation of appetite (Carter et al., 2020). Measuring endogenous oxytocin has potential to advance our understanding of the neurophysiological correlates of social behaviors (Crockford et al., 2014), predict responses to exogenous oxytocin as a therapeutic intervention (Parker et al., 2017), and characterize associations between circulating oxytocin concentrations and behavioral phenotypes or disease risks (Skrundz et al., 2011).

Despite widespread interest in measuring oxytocin, the best approaches for doing so remain debated, and common approaches to sample preparation and measurement yield uncorrelated results, raising questions about the validity and robustness of some current analytical techniques (Leng and Sabatier, 2016; MacLean et al., 2019). The most common approaches to oxytocin measurement involve competitive immunoassays, in which a fixed amount of labeled hormone and an unknown amount of native hormone in a biological sample compete for binding to an antibody. Although competitive immunoassays can be highly sensitive, they can also be susceptible to a wide range of problems. For instance, antibodies may react nonspecifically by binding to other proteins in a biological sample, resulting in a false positive signal. Recent studies demonstrate the potential for this phenomenon in oxytocin immunoassays. Specifically, oxytocin was falsely detected in plasma and urine samples from oxytocin knockout (OTKO) mice, with the extent of this effect varying by sample matrix, sample dilution, and across different commercially-available assays (Gnanadesikan et al., 2022, 2021). Although a range of sample extraction techniques have been developed to minimize potential interferences in oxytocin immunoassays, commonly used extraction protocols do not always effectively

remove interfering molecules, these approaches may induce additional forms of interference, bioactive metabolites of oxytocin can be discarded (which may or may not be desirable), and the potential for extraneous variation increases through the addition of preanalytical steps (Gan et al., 2023; Gnanadesikan et al., 2022).

One potentially promising approach for overcoming challenges to oxytocin quantitation involves measurement of neurophysin I (NP-1) – oxytocin’s carrier molecule – as a surrogate biomarker (Tabak et al., 2023). Oxytocin is enzymatically cleaved from NP-1 during axonal transport, after which both molecules are secreted in equimolar concentrations (Gimpl and Fahrenholz, 2001). However, although NP-1 is released in a 1:1 ratio with oxytocin, it circulates in much higher concentrations, potentially due to differences in the half-lives of oxytocin and NP-1. The half-life of NP-1 is approximately twice that of oxytocin in cerebrospinal fluid, although the difference in half-lives in blood, particularly at physiological concentrations, remains poorly understood (Morris et al., 1975; Watkins et al., 1975). In addition, due to its larger size (94 amino acids compared to 9 for oxytocin), NP-1 can be measured using a sandwich immunoassay, an approach that uses paired capture and detection antibodies which bind to non-overlapping epitopes on the antigen (Hayrapetyan et al., 2023). This approach affords greater sensitivity and specificity than competitive assays and is suitable for complex biological matrices (e.g., plasma), which can typically be analyzed without extraction.

The prospect of measuring NP-1 is not new, and indeed early studies of endogenous oxytocin release often also involved measurements of NP-1 (Amico et al., 1981). However, with the advent of multiple oxytocin immunoassays in the 1980s, measurement of NP-1 became much less common and is rarely used today. Recently, the strategy of measuring a larger and more stable product of the prepropeptide has been used effectively with the closely related molecule, arginine vasopressin. Specifically, copeptin – a cleavage product of preprovasopressin – has been introduced as a surrogate biomarker for vasopressin, as measurement of vasopressin is susceptible to many of the same challenges researchers face with oxytocin. Copeptin has proven to be a broadly useful biomarker for a wide range of diseases involving dysregulation of the vasopressin system (Morgenthaler et al., 2008). Measurement of NP-1 has potential to present similar advantages in studies of endogenous oxytocin, but this approach requires foundational validation studies which have yet to be conducted. Here, we report a series of experiments that 1) analytically validate a commercially available NP-1 sandwich immunoassay for use with human plasma and urine samples, 2) confirm the specificity of this assay for the oxytocin neurophysin, based on detection of NP-1 in plasma from wild-type but not oxytocin knockout mice, 3) demonstrate changes in circulating NP-1 concentrations in human mothers from late gestation to the postpartum period, and 4) establish strong correlation between plasma oxytocin and NP-1 concentrations in humans.

## Validation of a Neurophysin I Sandwich Immunoassay

We used a commercially available sandwich immunoassay distributed by Abcam (product number ab242248) for quantitation of NP-1. This kit has a reported detection range of 125-8000 pg/mL. We conducted an analytical validation to assess parallelism and spike

recovery using a human plasma and urine pool. To assess parallelism, we serially diluted unextracted plasma and urine samples using a 3:4 (part:whole) dilution series. Both plasma and urine samples diluted in parallel to the standard curve (Figure 1A, 1B) and the coefficient of variation (CV) for dilution-corrected concentrations was excellent in both cases (CVs, plasma = 10.0%; urine = 3.9%). Accuracy was assessed using a spike recovery procedure. Spiked samples consisted of 90% sample matrix (plasma at 1:2 dilution, urine at no dilution) and 10% synthetic NP-1 at five different concentrations (~50-800 pg/mL). Spike recovery was within recommended guidelines for both matrices (recovery, plasma: mean = 85%, range = 79-95%; urine: mean = 91%, range = 85-93%).

To assess specificity for NP-1, we measured plasma samples from male wildtype (C57BL/6J (B6)) and oxytocin knockout mice (Oxt<sup>tm1Zuk</sup>). This oxytocin knockout mouse model involves a deletion of exon 1 encoding the oxytocin peptide, including the start codon, preventing both oxytocin and NP-1 biosynthesis (Nishimori et al., 1996). Five plasma samples from wildtype, and six plasma samples from OTKO mice were measured at 1:8 dilution, with the person conducting the assay blind to genotype. All samples from OTKO mice measured below the lower limit of the assay's range whereas samples from wildtype mice yielded NP-1 concentrations within the assay's range in all cases (Figure 1C), confirming specificity for NP-1. Notably, however, we were unsuccessful in measuring NP-1 in dog plasma, even when samples were measured at 8x concentration, suggesting that one or both antibodies are sensitive to taxonomic differences in the NP-1 sequence (which unlike oxytocin, is highly variable across species).

## Neurophysin I Concentrations are Elevated in Late Pregnancy

Plasma samples from an ongoing clinical trial (NCT04818112; University of Arizona IRB protocol #2009050756) were used to assess NP-1 concentrations in human mothers (n = 30) from late pregnancy through three months following childbirth. Systematic reviews indicate that circulating oxytocin concentrations increase 3-4-fold across pregnancy in humans (Uvnäs-Moberg et al., 2019). We therefore expected that if NP-1 reliably captures this pattern of oxytocin secretion then we would observe the highest NP-1 concentrations in late pregnancy. Eighty plasma samples (collected into vacutainers containing ethylenediaminetetraacetic acid) from study participants, collected between 31-35 weeks of gestation, and 1-, 2-, and 3-months postpartum were assayed for NP-1 (1:2 dilution). One sample measured higher than the highest standard and was thus set to the highest measurable value (8,000 pg/mL) for purposes of analysis (this sample could not be remeasured because the remaining volume was required for additional analyses described below). NP-1 concentrations were, on average, ~6-fold higher in late gestation than in the months following childbirth (Figure 2A). There was substantial variation in NP-1 concentrations at each timepoint, with evidence for consistent individual differences across time. Pairwise Pearson correlation coefficients of log NP-1 concentrations at the four timepoints ranged from R = 0.40 to R = 0.91 (Figure 2B). The adjusted intraclass-correlation coefficient – a measure of repeatability – from a linear mixed model predicting log NP-1 concentrations as a function of study timepoint and a random intercept for participant ID was 0.58.

## Plasma neurophysin I and oxytocin concentrations (following solid-phase extraction) are highly correlated

To assess correlations between plasma NP-1 and oxytocin concentrations, we first created 20 plasma pools from the human samples described above. This pooling was performed to create adequate volume for measurement of oxytocin following two different preanalytical protocols. To create pools, samples were rank-ordered based on NP-1 concentrations and then combined in sets of 4 (e.g., the first pool consisted of the four samples with the highest NP-1 concentrations and the last pool consisted of the four samples with the lowest NP-1 concentrations). NP-1 concentrations for each pool were estimated as the mean concentration of the four individual samples comprising the pool. We used this approach to pooling samples to obtain the maximal range of variation in NP-1 concentrations against which to compare the corresponding oxytocin measurements.

Pools were measured for oxytocin using a competitive Enzyme-Linked Immunosorbent Assay (ELISA) manufactured by Arbor Assays (Catalog #K048). The epitope for the antibody in this assay spans oxytocin residues 2-9 (Gnanadesikan et al., 2022). We conducted oxytocin analyses following two commonly-used approaches to sample preparation: (1) *Solid phase extraction* – 950  $\mu$ L plasma was processed using a 1cc Hydrophilic-Lipophilic Balanced (HLB) cartridge (Oasis PRiME, Part no. 186 008 055, Waters Corporation), following a protocol described and validated by Gnanadesikan et al (2021). Samples were assayed at a 3.8x concentration. (2) *Unextracted* – neat plasma was diluted 1:2 (part:whole) in assay buffer before analysis (0.5x concentration). For oxytocin assays we retained measurements for statistical analysis if the coefficient of variation (CVs) for duplicate wells was < 30%, with the exception that we also retained measurements with higher CVs ( $n = 5$ ) if the mean measured concentration was below the assay's limit of detection (22.9 pg/mL). We implemented this strategy because these samples represented the lowest oxytocin measurements, which though imprecise in this region of the standard curve, were informative as low concentration samples when assessing correlations with NP-1. Sensitivity analyses excluding these samples did not substantively change our results.

Following solid phase extraction, plasma oxytocin concentrations were strongly, positively correlated with NP-1 concentrations ( $R = 0.84$ ,  $p < 0.001$ ; Figure 2C). In contrast, there was a weak, negative, and non-significant correlation between NP-1 and oxytocin measurements when the oxytocin assay was performed with unextracted plasma samples ( $R = -0.27$ ,  $p = 0.25$ ; Figure 2D). The difference between these correlations (assessed using the *cocor* R package, Diedenhofen and Musch, 2015) was statistically significant (Pearson and Filon's  $z = 4.62$ ,  $p < 0.0001$ ).

## Summary & Conclusions

Collectively, these studies suggest that measurement of NP-1 presents a promising approach for estimating (peripheral) endogenous oxytocin secretion, with many advantages relative to measuring oxytocin itself. The NP-1 assay used in this study has strong analytical properties (e.g., parallelism & accuracy), is specific for NP-1, does not require time-

intensive extraction protocols for use with human plasma or urine, and the assay itself can be completed in < 2 hours (compared to 16-24 hours for a competitive oxytocin immunoassay).

Plasma NP-1 concentrations were markedly elevated in late pregnancy, consistent with studies of oxytocin during gestation, and critically, we observed strong correlation ( $R = 0.84$ ) between plasma NP-1 and oxytocin concentrations, when plasma samples were processed using a solid-phase extraction. Importantly, however, many of the (extracted) oxytocin measurements yielded concentrations near or below the lower limit of detection for this assay, and many other commercially available oxytocin immunoassays, whereas all human NP-1 measurements produced results within the assay's range. Lastly, the lack of correlation between NP-1 and oxytocin concentrations when oxytocin was measured in non-extracted plasma provides further evidence that analysis of non-extracted plasma samples may be susceptible to inferences that compromise analytical results.

In sum, many of the limitations associated with current approaches to oxytocin measurement may be overcome by using NP-1 as a robust surrogate biomarker. The studies presented here demonstrate the promise of this approach, but further research employing parallel measurements of oxytocin and NP-1 in diverse populations and study designs will be essential. For example, it remains unknown whether NP-1 will be useful as a biomarker of acute oxytocin response, and further studies of the integration and clearance periods of NP-1 in different matrices will be required for biologically informed study designs. Key steps for the further development of these approaches will involve measurement of both oxytocin and NP-1 following exposure to stimuli known to induce oxytocin release (e.g., lactation, sexual stimulation, exercise). In these studies, it will be important to measure NP-1 in diverse biological matrices to determine the extent to which these measures correlate across the spectrum of biological samples currently used in oxytocin research, as well as potential differences in the time course of effects.

Like oxytocin, NP-1 is cleared primarily by the kidney and liver, where it is subject to degradation, and only a small fraction of NP-1 taken up by the kidney remains intact in urine (Morris et al., 1975). In preliminary experiments we have been unable to measure NP-1 in saliva samples, but future work is required to robustly assess the presence of NP-1 in additional sample types. Additionally, due to taxonomic variation in the NP-1 sequence, adoption of this approach in animal studies will likely require the development of additional assays with novel antibodies. Whereas the assay used here yielded immunoreactivity with both human and mouse samples (despite variation in their NP-1 sequences) it was not suitable for dogs. Researchers considering this assay for additional species may benefit by performing multiple sequence alignments to assess similarity with the mouse and human NP-1 sequence, but rigorous validation studies will be required in all cases to determine suitability for additional species. Although the full potential and limitations of these approaches remain to be determined, we hypothesize that measurements of NP-1 will provide a powerful tool in future oxytocin research.

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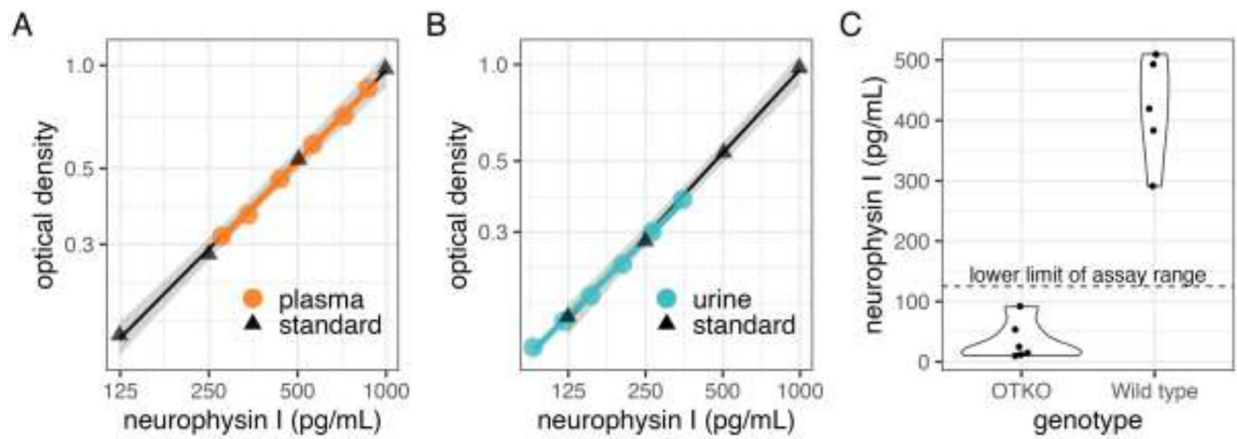
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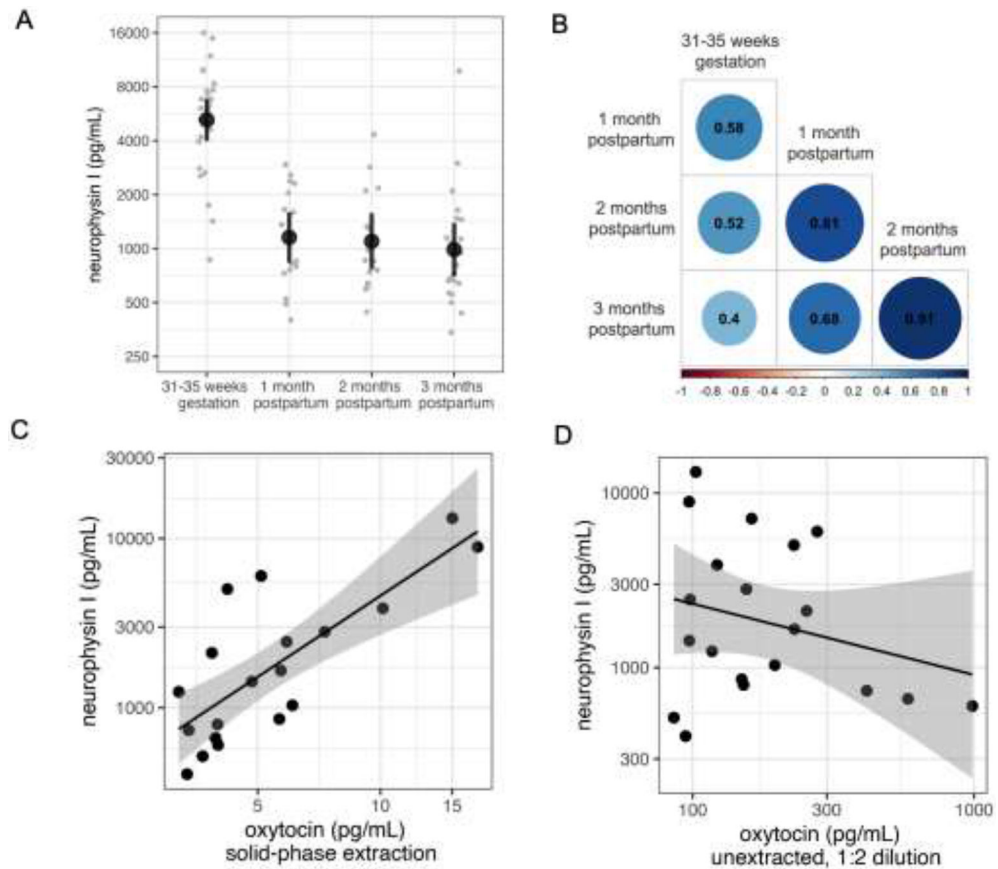
**Highlights**

- Analytical validation of a neurophysin I (NP-1) sandwich ELISA
- Specificity confirmed by NP-1 detection in wildtype but not oxytocin knockout mice
- Human plasma NP-1 concentrations are markedly elevated in late pregnancy
- Plasma NP-1 and oxytocin concentrations are highly correlated
- Neurophysin I (NP-1) is a robust surrogate biomarker for oxytocin



**Figure 1.**

Parallelism data for serial dilutions of (A) human plasma, and (B) human urine, followed by (C) measured NP-1 concentrations in wildtype and oxytocin knockout (OTKO) mice. In panels A and B, black triangles and regression lines represent serial dilutions of synthetic NP-1, and orange and blue circles and regression lines represent serial dilutions of plasma and urine samples, respectively. Concentrations in panel C reflect measured concentrations not corrected for sample dilution to enable visual comparison with the assay's range of detection (dashed horizontal line). The corrected NP-1 concentrations (only meaningful for measurements within the assay's range) range are 8x the plotted values in panel C.



**Figure 2.**

A) Neurophysin I (NP-1) concentrations in plasma samples collected from women in late pregnancy and 1-, 2-, and 3-months postpartum. Smaller, gray circles reflect individual sample concentrations. Larger black circles reflect means and 95% confidence intervals. B) Correlations between NP-1 concentrations in plasma samples across these four timepoints. C) Scatterplot of plasma NP-1 and oxytocin concentrations, when oxytocin samples are processed using a solid-phase extraction or D) measured without extraction at a 1:2 (part:whole) dilution. Plotted concentrations are corrected for dilution factor.