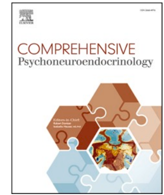
Comprehensive Psychoneuroendocrinology 15 (2023) 100188

Contents lists available at ScienceDirect

Comprehensive Psychoneuroendocrinology

journal homepage: www.sciencedirect.com/journal/comprehensive-psychoneuroendocrinology



Inaccuracies in plasma oxytocin extraction and enzyme immunoassay techniques

Hoong-Wei Gan [a,b,*], Clare Leeson [b], Helen Aitkenhead [b], Mehul Dattani [a,b]

[a] Genetics & Genomic Medicine Research and Training Department, UCL Great Ormond Street Institute of Child Health, 30 Guilford Street, London, WC1N 1EH, United Kingdom

[b] Great Ormond Street Hospital for Children NHS Foundation Trust, Great Ormond Street, London, WC1N 3JH, United Kingdom

ARTICLE INFO

Keywords:
Oxytocin
Enzyme-linked immunosorbent assay
Reverse-phase chromatography
Solid phase extraction

ABSTRACT

Numerous studies have reported extensive associations between plasma oxytocin (OXT) concentrations and various human physiological and neurobehavioral processes. Measurement of OXT is fraught with difficulty due to its low molecular weight and plasma concentrations, with no consensus as to the optimal conditions for pre-analytical sample extraction, standards for immunoassay validation or the ideal protease inhibitors to prevent OXT degradation. Previous attempts at determining the efficacy of various purification techniques such as solid phase extraction (SPE) or ultrafiltration have only utilized human plasma samples, making it difficult to dissect out whether the effect of interference comes from the extraction process itself or cross-reactivity with other proteins. By testing these on pure OXT solutions, we demonstrate poor recovery efficacy and reliability of reversed phase SPE (maximum 58.1%) and ultrafiltration (<1%) techniques, and the potential for the former to introduce interference into enzyme immunoassay (EIA) measurements. The clonality of antibodies used in EIA kits also potentially contributes to the differences in the readings obtained, and we validate an EIA kit which did not require pre-analytical sample extraction with low cross-reactivity and high reliability (intraclass correlation coefficient 0.980 (95% CI 0.896–0.999). Biochemical techniques used for measuring plasma OXT concentrations must therefore be internally validated prior to translation into clinical studies.

1. Introduction

As a nonapeptide present in concentrations 100 million to ten billion times lower (pg/ml vs. g/l) than albumin in human plasma, measuring the concentrations of the neurohypophyseal hormone oxytocin (OXT) accurately has proven difficult, with a lack of consensus as to the optimum assay conditions that should be used [1–3]. The cheapest, most widely available method of analysis involves the use of radio- (RIA) or enzyme-based immunoassays (EIA). However, outside of the variability in sensitivity and specificity of these different assays, other pre-analytical factors can also significantly affect the measured concentration of human plasma OXT.

Firstly, OXT has a short half-life in human plasma due to the activity of proteases, the most well-described being the widely expressed oxytocinase (a leucine/cysteine aminopeptidase), which cleaves the N-terminal cysteine from the adjacent tyrosine residue [4]. In pregnancy, it is secreted into plasma to modulate OXT concentrations and prevent premature delivery. In order to increase the stability of OXT in plasma, a wide variety of protease inhibitors have been used with no consensus as to what is optimum or, indeed, if they should be used at all. To date, only one study has directly compared the efficacy of different protease inhibitors in inhibiting breakdown of OXT in human plasma, even then finding that the use of protease inhibitors could in themselves cause interference of radioimmunoassays [5]. The commonest inhibitor in use in many plasma OXT quantification studies is the serine protease inhibitor aprotinin/trasylol.

Abbreviations: OXT, Oxytocin; EIA, Enzyme immunoassay; RIA, Radioimmunoassay; SPE, Solid phase extraction; ACN, Acetonitrile; TFA, Trifluoroacetic acid; H_2O, Water; EDTA, Ethylenediaminetetracetic acid; AChE, Acetylcholinesterase; AVP, Arginine-vasopressin; SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis; IVIG, Intravenous immunoglobulin; HSA, Human serum albumin; DTT, Dithiothreitol; PVDF, Polyvinylidene difluoride; ICC, Intraclass correlation coefficient; ANOVA, Analysis of variance; CV, Coefficient of variation; LC-MS, Liquid chromatography - mass spectrometry; PBS, Phosphate-buffered saline.

* Corresponding author. Great Ormond Street Hospital for Children NHS Foundation Trust, Great Ormond Street, London WC1N 3JH, United Kingdom.

E-mail addresses: hoong.gan.11@ucl.ac.uk (H.-W. Gan), clareleeson.jobs@gmail.com (C. Leeson), Helen.Aitkenhead@gosh.nhs.uk (H. Aitkenhead), m.dattani@ucl.ac.uk (M. Dattani).

https://doi.org/10.1016/j.cpnec.2023.100188
Received 16 August 2022; Received in revised form 1 June 2023; Accepted 4 June 2023
Available online 8 June 2023
2666-4976/© 2023 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Secondly, due to its low molecular weight and low concentrations in human plasma, various sample extraction techniques to reduce the interference of other larger, more abundant proteins and the effect of plasma protein binding have commonly been used in the quantification of plasma OXT. These techniques are often combined with a concentration step, thereby increasing the concentration of OXT in the plasma sample to within the measurable range of the intended assay. Once again, there has been no consensus as to which is the best technique for this purpose, with OXT extraction processes generally falling into three categories: plasma chromatography-based techniques (solid phase extraction (SPE)), liquid-liquid extraction and ultrafiltration.

Most authors use "reversed phase" SPE, where OXT is eluted with a solvent of lower polarity to plasma (e.g. acetonitrile) from a relatively non-polar, hydrophobic stationary phase (e.g. C-18) where it has been non-covalently bound. Contrastingly, ultrafiltration involves the use of a membrane with pore sizes which only permit substances below a particular molecular weight limit to pass through into the filtrate, whilst higher molecular weight substances remain in the retentate. However, the necessity of the extraction process is also widely debated [1–3,6–8] and there are equivalent numbers of studies measuring plasma OXT with (normal range typically <1–100 pg/ml) and without (normal range typically 10-1000 pg/ml) plasma extraction [8–13]. Importantly, most of these studies have not tried to elucidate whether OXT can even be effectively extracted from pure solution using these techniques. Only one report by Gnanadesikan *et al.* (2022) [14] attempted to do this and achieved an extraction efficiency of 83–92% using a mixed-mode cation exchange method.

Lastly, although there are now numerous published studies on the role of OXT in a wide range of human physiological and neurobehavioral processes apart from its classical roles in parturition and lactation, the immunoassays used in many of these reports are not usually subject to robust internal validation prior to utilisation. Some authors have suggested that RIA-measured plasma OXT concentrations seem less dependent on plasma extraction processes than those derived from EIAs [8,15], with multiple potential explanations apart from the variable specificities of the antibodies used in these assays, including matrix effects on the enzyme, and the potential steric effect of the enzyme on OXT. Compared to EIAs, RIAs require laboratory facilities able to handle radioisotopes. Similarly, more recently developed mass spectrometry techniques [6,16–18] also require specialized equipment which is not widely available. EIAs therefore remain the most common assay technique in use.

This study therefore aimed to determine the optimal conditions for the measurement of human plasma OXT concentrations by examining the efficacy and reliability of two plasma extraction techniques (reversed phase SPE and ultrafiltration) in extracting OXT from plasma, the validity and reliability of two commercially available plasma OXT EIAs, and the role of aprotinin in maintaining the stability of OXT in human plasma prior to analysis.

2. Materials and methods

Ethical approval

The research protocol received ethical approval from the National Research Ethics Service Committee (London – Bloomsbury) on December 12, 2013 (REC reference 13/LO/1611) as part of a wider clinical study. All experiments were performed in accordance with the principles set out in the Research Governance Framework for Health and Social Care (Department of Health, 2005) with oversight by the Great Ormond Street Hospital for Children/University College London Great Ormond Street Institute of Child Health Joint Research and Development Office. All human controls provided informed consent prior to donating their samples for these experiments.

2.1. Preparation of pure OXT solutions

OXT peptide (Tocris Bioscience, Bristol, UK; catalog no. 1910 batch #13A) was dissolved in ultrapure water to a net concentration of 1 mg/ml. The manufacturer's product data sheet indicates a purity of >97% with 87.5% net peptide. Verification experiments were performed which confirmed a mean net peptide content of 83.6% (data not shown). All further calculated OXT concentrations were based on this value. The solution was then divided into 5 μl aliquots, evaporated to dryness in a centrifugal concentrator under vacuum and stored at −20 °C until analysis.

2.2. Preparation of blood samples

Blood samples were drawn from four (two female, two male) healthy adult volunteers into chilled ethylenediaminetetracetic acid (EDTA) tubes containing 400 kIU/ml aprotinin (Trasylol®, Nordic Pharma Ltd., Reading, UK) and centrifuged at 1600*g* for 15 min at 4 °C. The plasma supernatant was saved and frozen at −80 °C until analysis. For experiments on OXT stability in human plasma, aprotinin was added to only half the plasma sample drawn (see Section 2.8).

2.3. Reversed phase SPE protocol

3 ml (200 mg) Sep-Pak® C18-bonded silica columns (Waters Corporation, Massachusetts, USA) were equilibrated with 1 ml of 100% acetonitrile (ACN), followed by 10 ml of 0.1% trifluoroacetic acid (TFA) in ultrapure water (TFA-H_2O). 1 ml of the sample of interest (either OXT peptide or plasma) was diluted 1:1 with 0.1–0.5% TFA-H_2O binding buffer and centrifuged at 17000*g* for 15 min at 4 °C. The supernatant was then loaded onto the C-18 columns by gravity-feeding, after which the columns were washed with 10 ml of 0.1% TFA-H_2O. Elution was performed by applying 1–3 ml of 15–95% ACN in 0.1–0.5% TFA-H_2O. Aliquots of the load, wash and elute fractions were evaporated to dryness in a centrifugal concentrator under vacuum and stored at −20 °C until analysis, whereupon they were reconstituted in EIA buffer and used immediately.

2.4. Ultrafiltration protocol

Ultrafiltration was performed based on the method described by Ref. [19]. 2 ml of sample was loaded onto Amicon® Ultra centrifugal filters with nominal molecular weight limits of 3 and 10 kDa (Merck Millipore, Massachusetts, USA) and then centrifuged at 4000*g* for 60 min at 4 °C. The ultrafiltrate was collected and the device inverted and centrifuged at 1000*g* for 2 min at 4 °C to obtain the retentate. Aliquots of both the ultrafiltrate and retentate were evaporated to dryness in a centrifugal concentrator under vacuum and stored at −20 °C until analysis, whereupon they were reconstituted in EIA buffer and used immediately.

2.5. EIAs

Two commercially available EIA kits commonly used in published literature were chosen for validity and reliability assessments, due to significant differences in the nature of the competitive EIA. Both assays contain a primary polyclonal rabbit anti-OXT IgG. The assay by Enzo Life Sciences (New York, USA) utilizes a secondary polyclonal goat anti-rabbit IgG and an alkaline phosphatase/*p*-nitrophenylphosphate (*p*NPP) reaction, whilst the assay by Cayman Chemical Company (Michigan, USA) utilizes a secondary monoclonal mouse anti-rabbit IgG and an acetylcholinesterase (AChE)/acetylthiocholine/5,5'-dithio-*bis*-(2-nitrobenzoic acid) reaction. The published IC_{50} for the Enzo Life Sciences assay was 228 pg/ml with a lower limit of detection (80% B/B_0) of 43 pg/ml and sensitivity of 15.0 pg/ml, whilst for the Cayman Chemical Company assay the mean IC_{50} was 80 pg/ml with a lower limit of

detection of 18 pg/ml and sensitivity of 5.9 pg/ml. All standards (as provided in the respective kits) and samples were assayed in triplicate and calculation of OXT concentrations was performed using BioTek Gen5 data analysis software (BioTek Instruments Inc., Vermont, USA) with a four-parameter logistic fit. The manufacturers of the Cayman Chemical Company assay expressly stipulate that aprotinin should not be used in plasma samples used for analysis, however, to be able to directly compare measurements between the two kits using the same plasma samples, all EIAs utilising plasma samples included control wells containing pure aprotinin as a negative control.

2.6. Validity testing

Validity testing of the EIAs was performed by conducting spike recovery, dilutional linearity and cross-reactivity experiments. Parallelism studies were not possible due to the low OXT concentrations [20]. Spike recovery was assessed by adding small volumes (<2% of total volume) of neat EIA standard solution at concentrations of 200 and 400 pg/ml to human plasma samples from four healthy volunteers (2 female, 2 male) and assay buffer. Dilutional linearity was assessed by adding a small volume (<1% of total volume) of neat EIA standard solution to plasma from a healthy human male volunteer to a concentration of 700 pg/ml and serially performing 1:2 dilutions with EIA buffer to a final dilution of 1:16. Cross-reactivity was performed using the 19 most abundant proteins in human plasma (Supplementary Tables A1), arginine-vasopressin (AVP) and aprotinin at concentrations of 3 μg/ml and 1 μg/ml for kits 1 and 2 respectively (based on a target cross-reactivity (IC_{50}(OXT)/IC_{50}(interfering protein)) of <0.01%). Interfering proteins resulting in detectable readings on the OXT EIA were subject to further analysis using 10-fold dilutions between 100 ng/ml to 1 mg/ml to determine their exact IC_{50}.

Further analysis of the specificity of EIA antibodies was conducted by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Briefly, samples of human plasma, intravenous immunoglobulin (IVIG) and human serum albumin (HSA) containing 20 μg of protein in 1:2 sample buffer (Novex® Tricine SDS (Life Technologies, California, USA)) underwent SDS-PAGE at 125V for 60 min, with or without a pre-loading reduction step with 50 mM dithiotreitol (DTT) and/or heating of the samples at 85 °C for 2 or 10 min. Following protein transfer to polyvinylidene fluoride (PVDF) membranes, Western blotting was carried out with or without the primary polyclonal rabbit anti-OXT IgG from kits 1 and 2 (1:100) and secondary polyclonal goat anti-rabbit (1:80,000) or monoclonal mouse anti-rabbit (1:320,000) IgG before development.

2.7. Precision testing

Intra-assay precision was tested by assaying 19 replicates of unextracted plasma from three healthy human controls (two female, one male) on the same EIA plate. The minimum sample size required for an estimated intraclass correlation coefficient (ICC) of 0.9 ± 0.2 for 19 replicates in a one-way random effects model was 2.7.

Inter-assay precision was tested by assaying unextracted plasma from four healthy human controls (two female, two male) on three separate EIA plates on separate days in triplicate. The minimum sample size required for an estimated ICC of 0.9 ± 0.2 for three separate assays in a two-way random effects model was 3.6.

2.8. OXT stability studies

The effect of aprotinin on the stability of plasma OXT was assessed by collecting a single plasma sample from a healthy human male volunteer and adding 400 kIU/ml aprotinin to half of the sample. The samples were kept at room temperature and aliquots of plasma were frozen down daily for 10 days at −80 °C. These aliquots were then thawed simultaneously and OXT concentrations were subsequently measured by EIA using the Cayman Chemical Company kit at the end of this period.

2.9. Statistical analysis

Where more than one repeat experiment was performed, plasma OXT concentrations obtained by EIA were expressed as mean ± SD. Comparisons between different OXT measurements using different pre-analytical and EIA techniques were analyzed using the student's t-test, or a one-way analysis of variance (ANOVA). The Shapiro-Wilk test was used to confirm the normality of data. ICCs were calculated using a one-way random effects model for intra-assay precision and a two-way random effects model for inter-assay precision. All statistical analyses were performed using SPSS version 25.0 (IBM, Armonk, New York, USA), and all outlier data points were included, as the analysis was aimed at determining the reliability of assay readings.

3. Results

3.1. Reversed phase SPE of OXT from pure solution

Initial experiments to determine optimal reversed phase SPE conditions to achieve maximal extraction efficacy for OXT were conducted using pure OXT peptide solution and the Enzo Life Sciences kit (a commonly used EIA assay) to elucidate the behavior of OXT in the absence of other interfering plasma substances. Initial experiments using 200 pg/ml pure OXT solution, a single C-18 column gradient elution of 5–95% ACN (in 1 ml 5% increments) and 0.1% or 0.5% TFA-H_2O as binding and eluting buffer, showed firstly that 0.5% TFA-H_2O resulted in unusually high OXT concentrations in the eluate fractions, such that the total measured OXT in all fractions (1251.9 pg) was higher than that initially loaded, suggesting an element of interference from the extraction process itself. Contrastingly, using 0.1% TFA-H_2O, the total OXT present in all fractions was 79.1 pg, with only 37.7 pg being eluted out entirely between 15 and 40% ACN eluates, giving an overall recovery efficiency of only 18.9% (Fig. 1a). Subsequent experiments using 200 pg/ml pure OXT solution with 0.1% TFA-H_2O and separate C-18 column elutions with 15, 20, 25, 30, 35 and 40% ACN revealed that maximum OXT recovery was achieved in the 30–40% ACN range, but with only 10.1–49.1% of OXT recovered in the eluate, with high coefficients of variation (CV for three columns of the same ACN concentration 30.8–136.5%). We also analyzed the load and wash fractions for OXT and found that the total amount of OXT measured in all fractions was still only 37.1–92.2% of the original sample, indicating significant OXT remaining on the C-18 columns and poor reproducibility (illustrative Fig. 1b).

Subsequent experiments attempting to further optimize the reversed phase SPE process, including using larger eluent volumes (3 ml) in a single column with partial sequential gradient elution of 30–40% ACN, or separate columns with 30, 35 and 40% ACN showed that recovery efficacy could only be increased slightly using a single 3 ml elution with 35% ACN to a maximum of 58.1% (Fig. 1c, CV 10.3%). Further attempts at trying to reduce OXT losses during the wash steps by recycling and reloading the wash fractions onto the C-18 columns did not improve this (data not shown).

3.2. Ultrafiltration of OXT from pure solution

Given the above difficulties, further experiments were conducted using ultrafiltration to see if this would improve the recovery of OXT. However, recovery of OXT using both 3 kDa and 10 kDa filters was extremely poor, with readings all below the sensitivity of the assay, giving recovery efficacies of <1% (Fig. 1d).

3.3. Validity testing of EIAs

Given the poor recovery of OXT from pure solution by reversed phase

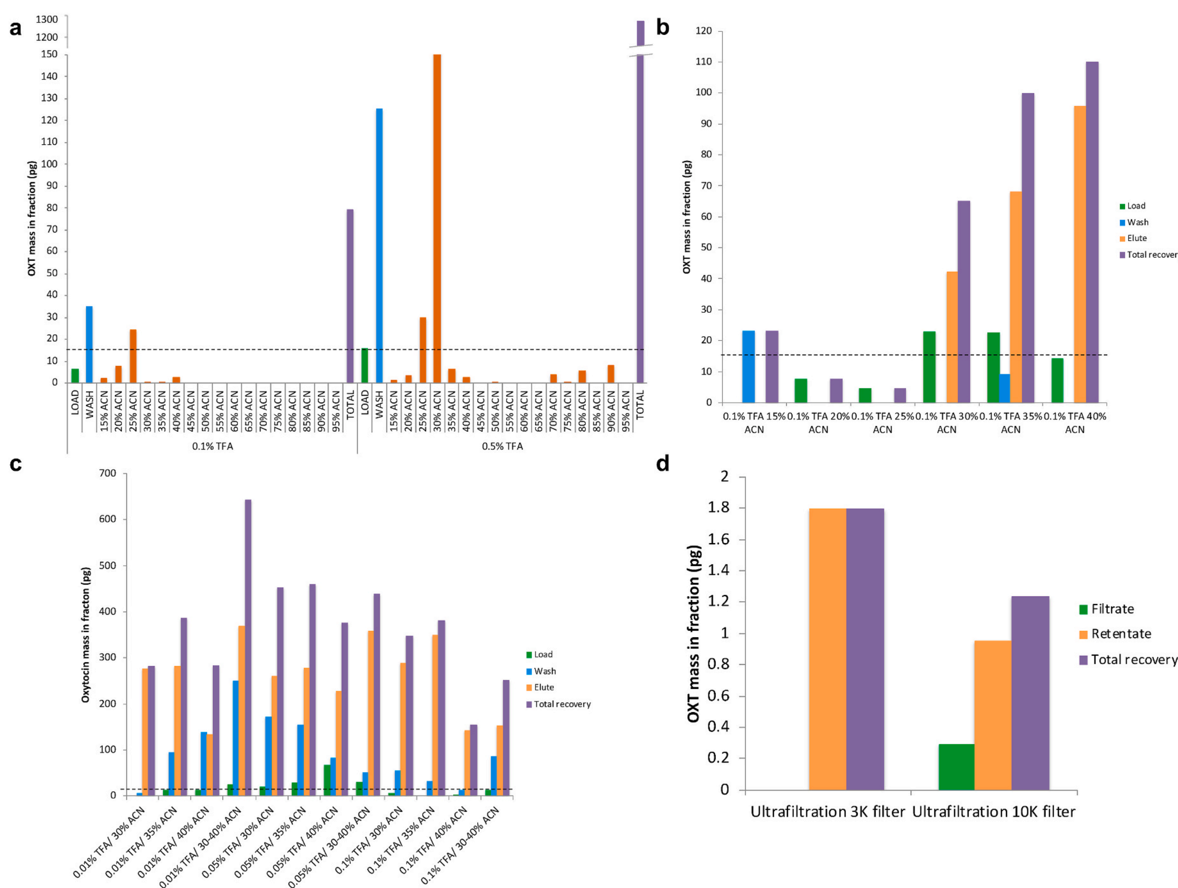


Fig. 1. Plasma extraction of OXT by (a–c) solid phase extraction (SPE) and (d) ultrafiltration. Horizontal dashed lines indicate the sensitivity of the assay (Enzo Life Sciences). Note that the measured quantities of OXT below this limit may not be completely reliable. (a–b) SPE of 200 pg of pure OXT solution using (a) a single C-18 column gradient elution of 15–95% acetonitrile (ACN) with 0.1–0.5% trifluoroacetic acid/water (TFA-H_2O), and (b) separate C-18 column elutions of 15, 20, 25, 30, 35 and 40% ACN with 0.1% TFA-H_2O. Note that in (a) the bars representing the quantity of OXT measured in the 0.5% TFA/30% ACN eluate (1049.2 pg) and the total OXT recovery (1251.9 pg) have been foreshortened to permit display of the rest of the chart as they extend much higher than the upper limit of the y-axis. (c) SPE of 600 pg of pure OXT solution using separate C-18 column elutions of 3 ml 30, 35, 40% ACN and a gradient elution of 30–40% ACN with 0.01–0.1% TFA-H_2O. The measured unextracted OXT in this series of experiments was 663 pg (not shown). (d) Ultrafiltration of 100 pg/ml pure OXT solution with 3 kDa and 10 kDa centrifugal filters. Note the significant under-recovery in all cases, all of which were below the sensitivity of the assay.

SPE and ultrafiltration, a second EIA assay (Cayman Chemical Company) was examined to compare its performance to the Enzo Life Sciences kit in measuring OXT concentrations from both extracted and unextracted pure solution (600 pg/ml) and plasma samples from a healthy male volunteer (Fig. 2). Briefly, 0.1% TFA-H_2O was used in the load and wash steps, with recycling of the wash fraction once, followed by a single column gradient elution using 2 ml each of 30, 35 and 40% ACN (total 6 ml). Whilst recovery efficacy from pure OXT solution remained poor when analyzed by either kit (Enzo Life Sciences 9.3% vs Cayman Chemical Company 41.0%), interestingly, with regard to human plasma, the total apparent OXT recovered from all fractions (load, wash, eluate) was higher than that of unextracted plasma (Enzo Life Sciences: mean extracted plasma total recovery 1514.7 pg vs. unextracted plasma 767.86 pg, Cayman Chemical Company: mean extracted plasma total recovery 784.6 pg vs. unextracted plasma 43.7 pg). Additionally, the measured OXT concentrations in the final eluate for extracted vs. unextracted plasma were significantly different for the Enzo Life Sciences kit (54.6 ± 1.0 pg/ml vs 767.9 ± 39.9 pg/ml, $p < 0.0001$) but not the Cayman Chemical Company kit (53.8 ± 8.9 pg/ml vs. 43.7 ± 23.0 pg/ml, $p=0.7$). Together, these data suggest that the

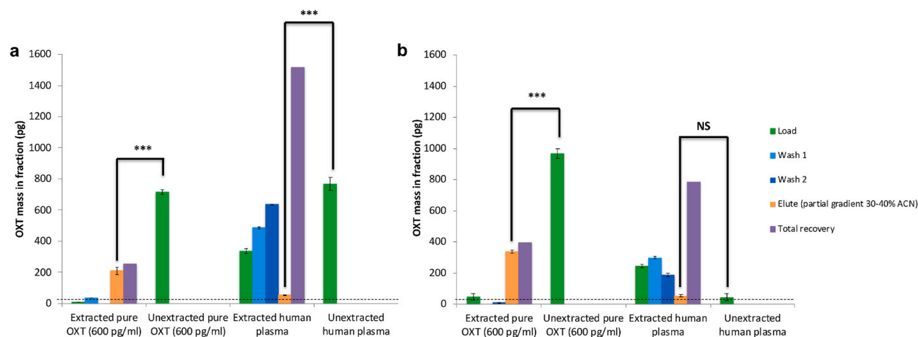


Fig. 2. Comparison of the performance of the Enzo Life Sciences kit (a) vs. the Cayman Chemical Company kit (b) in the analysis of 1 ml extracted and unextracted samples of 600 pg/ml pure OXT solution and human plasma from a healthy male volunteer. Horizontal dashed lines indicate the sensitivity of the assays. SPE of plasma OXT was carried out using a 6 ml gradient elution of 30–40% ACN with 0.1% TFA-H_2O, with recycling of the wash fraction once. Results are mean ± SD of three experiments. ***$p < 0.001$; NS, non-significant.

Enzo Life Sciences kit was less specific for measuring human plasma OXT concentrations and/or that the SPE process itself may introduce interference in the final eluate. Indeed, loading of a pure 0.1% TFA-H_2O sample onto an Enzo Life Sciences EIA plate resulted in a measured "OXT" concentration of 6.5 ± 1.1 pg/ml. Notably this would have excluded a drying/evaporation step which may have altered the final pH of the sample. Additionally OXT concentrations measured using the Cayman Chemical Company kit did not appear to require a pre-analysis extraction step.

The two kits were further validated using cross-reactivity studies using the 19 most abundant proteins in human plasma, AVP and aprotinin (Table 1). In comparison to the Cayman Chemical Company kit, the Enzo Life Sciences kit showed a high degree of immunoreactivity with 21 of 22 of the proteins tested, three of which were near or above the sensitivity of the assay (ApoAII, C1q, AVP). For the Cayman Chemical Company kit, the IC_{50} of the four proteins exhibiting any immunoreactivity (HSA, IgG, IgM and transferrin) was >1,000,000 pg/ml, i.e. a cross-reactivity of <0.01% (Fig. 3). In order to determine the mechanism behind these differences in specificity, Western blotting of human plasma, intravenous immunoglobulin (IVIG) and HSA was performed utilising the primary polyclonal rabbit anti-OXT IgG, and the secondary polyclonal goat anti-rabbit (Enzo Life Sciences) or monoclonal mouse anti-rabbit (Cayman Chemical Company) IgGs from each kit. Indeed, the primary antibodies and monoclonal secondary antibody exhibited no interaction with either of these potentially interfering proteins, whilst the secondary polyclonal antibody showed direct interaction with IgG which was only disrupted by heating the protein samples to 85 °C for 10 min (Fig. 4). This suggested that the difference in specificity was at least partially due to the clonality and non-specificity of the secondary antibody used in the Enzo Life Sciences kit.

Further experiments were therefore conducted to validate the accuracy of the Cayman Chemical Company kit. Mean spike recovery from unextracted plasma from four healthy human volunteers was 131.3 ± 15.9% and 95.0 ± 23.9% for spiked concentrations of 200 pg/ml and 400 pg/ml pure OXT solution. Dilutional linearity ranged from 112 to 202% (dilution 1:1 to 1:16) for a spiked concentration of 700 pg/ml into unextracted plasma from a healthy human male volunteer, with a strong positive correlation between measured OXT concentration and dilution (R^2=0.99954, p=0.0002, Supplementary Figure A1).

Table 1
Cross reactivity readings using 3 μg/ml (Enzo Life Sciences kit) and 1 μg/ml (Cayman Chemical Company kit) concentrations of the 19 most abundant proteins in human plasma, AVP and aprotinin in an OXT EIA. The difference in concentrations used between the two EIAs was due to the difference in IC_{50} for the two kits. Results are mean ± SD of triplicate readings. *Only one reading was obtained, the rest of the measurements in triplicate were undetectable.

Protein	Kit 1 (pg/ml)	Kit 2 reading (pg/ml)
Albumin	1.5 ± 2.1	3.4 ± 0.2
Immunoglobulin G	10.1 ± 7.4	1.2*
Immunoglobulin A	1.8 ± 0.2	Undetectable
Immunoglobulin M	1.9 ± 1.4	0.8*
Transferrin	10.7 ± 7.9	0.05*
Fibrinogen	5.5 ± 4.5	Undetectable
α_2-macroglobulin (α2M)	2.1 ± 1.1	Undetectable
α_1-antitrypsin (α1AT)	0.5 ± 0.4	Undetectable
Haptoglobin	1.5 ± 1.4	Undetectable
α_1-acid glycoprotein (α1AG)	3.9 ± 2.3	Undetectable
Caeruloplasmin	2.2 ± 0.5	Undetectable
Apolipoprotein A-I (ApoAI)	3.3 ± 2.4	Undetectable
Apolipoprotein A-II (ApoAII)	14.7 ± 3.0	Undetectable
Apolipoprotein B (ApoB)	3.5 ± 0.8	Undetectable
C1q	23.1 ± 3.2	Undetectable
C3	4.4 ± 2.3	Undetectable
C4	Undetectable	Undetectable
Plasminogen	1.6 ± 1.1	Undetectable
Prealbumin	1.8 ± 2.4	Undetectable
Arginine-vasopressin (AVP)	25.2 ± 2.9	Undetectable
Aprotinin	0.9 ± 0.4	Undetectable

3.4. Precision testing of the Cayman Chemical Company kit

The intra-assay ICC for 19 single-plate replicate measurements of OXT concentrations in plasma from three healthy human volunteers was 0.999 (95% CI 0.998–1000, F(2,54)=1595.742, p < 0.000001), whilst the inter-assay ICC for three separate measurements of OXT concentrations in plasma from four healthy human volunteers on different EIA plates on different days was 0.980 (95% CI 0.896–0.999, F(3,6)= 140.234, p=0.000006). The mean OXT concentrations and CVs for each of these experiments is detailed in Supplementary Tables A2 and A.3.

3.5. Effect of aprotinin on OXT stability

Aprotinin significantly reduced the rate of OXT degradation, but only within the first 24 h, following which OXT concentrations remained relatively stable up to day 10 (Fig. 5).

4. Discussion

Despite the vast body of literature on the role of OXT in various aspects of human physiology and behavior, many of these studies do not report whether internal validation of the pre-analytical steps and immunoassays used has occurred prior to translation into clinical studies. Studies specifically reporting plasma or serum OXT concentrations in healthy human participants utilize widely varying pre-analytical sample processing methods, protease inhibitors, sample extraction processes and immunoassays. As a result, it is unsurprising that the "normal range" of human plasma OXT concentrations previously reported is wide. Here, we present data illustrating the significant difficulties in achieving consistent, efficacious extraction of OXT as well as highlighting the need for robust internal validation of any commercially purchased immunoassay kit prior to use.

Prior to this, there have been few studies analysing the behavior of OXT in pure solution when put through the same extraction processes used to purify human plasma. Without determining this, it becomes impossible to dissect whether the measured OXT concentrations in the final extracted samples are affected by residual interfering plasma proteins due to poor extraction efficacy and immunoassay cross-reactivity, or by other interfering substances introduced into the sample by the extraction process itself. As a result, reported spike recovery rates for various human plasma purification processes have typically been higher than those calculated from this study (normal phase chromatography 39–100% [15,21,22], reversed phase chromatography 65–108% [23–25], liquid-liquid extraction 61–143% [8,23,26], ion exchange chromatography 65% [27], high performance liquid chromatography (HPLC) 87–95% [8,28], ultrafiltration 68–92% [19,29]). With particular reference to techniques relying on the relative solubility of OXT in hydrophobic/hydrophilic solutions such as SPE and liquid-liquid chromatography, it is important to note that the OXT peptide is neither predominantly hydrophobic or hydrophilic, with four of its nine amino acid residues (isoleucine, proline, leucine and glycine) being hydrophobic, whilst the remaining five (cysteine, tyrosine, glutamine, asparagine) are hydrophilic [30]. Pre-acidification with TFA alters the polarity of OXT and attempts to optimize its interaction with the SPE columns, but even then this is inconsistent as evidenced by our experiments here.

Unsurprisingly, therefore despite numerous attempts at recovering OXT from pure solution using reversed SPE under different conditions, recovery efficiency was highly inconsistent, ranging from 10.1 to 58.1%. Importantly, the optimum concentration of ACN for elution of OXT from C-18 (30–40%) was much lower than that recommended by the kit manufacturers or described in the literature (60–95%) [8,9,31–33], and more in keeping with the recent findings of Gnanadesikan *et al.* (2021) [22] (20%–30%).

In some of these experiments, the total amount of OXT measured in all fractions of the SPE process exceeded that loaded onto the C-18

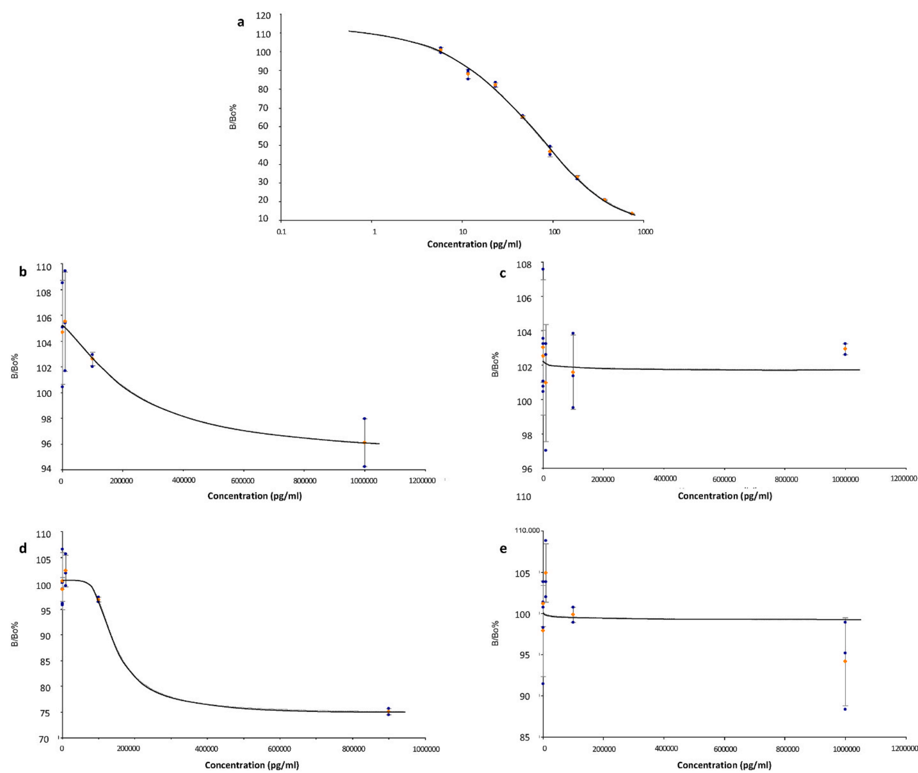


Fig. 3. EIA calibration curves for (a) oxytocin, (b) human albumin, (c) immunoglobulin G, (d) immunoglobulin M and (e) transferrin, using the Cayman Chemical Company kit. Note that even at the highest sampled concentration of 1 μg/ml, IC_{50} was not achieved for any of the interfering peptides, resulting in a cross-reactivity of <0.01%.

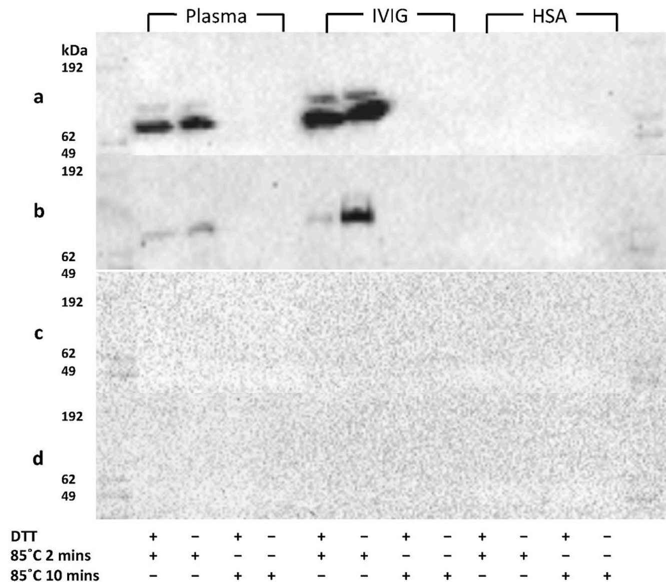


Fig. 4. Western blot results of human plasma, immunoglobulin G (IVIG) and human serum albumin (HSA) in the presence of (a–b) polyclonal goat anti-rabbit antibody and (c–d) monoclonal mouse anti-rabbit antibody, (a,c) with and (b,d) without the presence of polyclonal rabbit anti-OXT antibody, performed under various reducing/denaturing conditions. DTT, dithiotreitol.

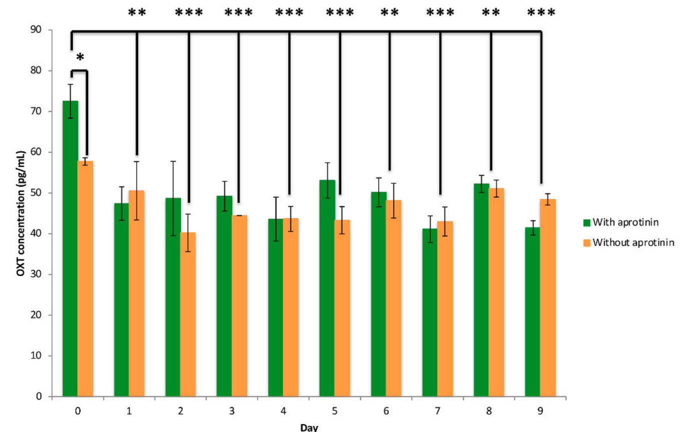


Fig. 5. Stability of OXT in human plasma, with (green bars) and without (orange bars) the presence of 400 kIU/ml aprotinin. After the first 24 h at room temperature, OXT concentrations did not decrease significantly further. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

column. In the complete absence of any other interfering substances, only the extraction process itself had the potential to introduce interference which was detectable on the EIA, and would be keeping with the high CVs observed in many of these assays as it is likely this interference is inconsistent across columns. This means that the true recovery efficacy in this series of experiments was even lower than this. This is a significant finding given that our initial experiments were performed on pure OXT solutions and may explain why some studies have found positive correlations between extracted and unextracted plasma OXT concentrations [14,22], whilst others have not [8,15].

Importantly, although Gnanadesikan *et al.* (2021) [22] reported extracted plasma OXT concentrations below the sensitivity of the assay using the Enzo Life Sciences kit in $Oxt^{-/-}$ knockout mice, it is still impossible to distill if the final readings obtained in their experiments were affected by introduction of interference by the SPE process utilized, which was similar to ours. In experiments conducted after our findings

in this study, the same authors achieved a lower recovery efficiency of 83–92% using pure OXT solutions and a mixed-mode cation exchange method which allowed for both the pH and hydrophilicity of the extraction columns to be manipulated [14]. This lower recovery efficiency compared to that obtained by the same authors previously [22] supports the hypothesis that certain SPE techniques can result in additive interference and artificially increased extraction efficiencies. Further work needs to be done to characterise this interference, ideally with the assistance of LC-MS methods.

Interestingly, further attempts to achieve a higher recovery efficacy using ultrafiltration were also unsuccessful, with recovery of OXT being even poorer at <1% and significant amounts of OXT presumably being retained on the ultrafiltration membrane. This finding was in marked contrast to the only two other studies documenting OXT recovery rates of 91.8% from plasma [19] and 68.4% from saliva [29] utilising the same ultrafiltration columns. Poor recovery from ultrafiltration has previously been described, and has been attributed to non-specific binding of the analyte of interest to the membrane, or variability in the purported membrane pore size [34]. It is also worth noting that ultrafiltration columns are usually designed to remove smaller molecular weight molecules from the sample in order to purify and concentrate higher molecular weight substances in the retentate rather than the filtrate.

Given the difficulties in achieving optimal reversed phase SPE conditions with sufficient recovery of OXT, a second EIA was selected for validation with a significantly different format to that of the Enzo Life Sciences kit. Comparison of the performance of both EIAs in the measurement of OXT concentrations from both extracted and unextracted pure solution and human plasma showed that the results obtained from the Cayman Chemical Company kit were not dependent on plasma extraction. Furthermore, cross-reactivity studies using the 19 most abundant proteins in human plasma, as well as AVP (given the similarity in structure to OXT) and aprotinin showed significant differences in the specificity of the two assays. This was further supported by Western blotting studies demonstrating that the polyclonal secondary antibody used in the Enzo Life Sciences kit could also bind to IgG, an interaction which was only disrupted by complete denaturation of IgG by exposure to prolonged, high temperatures. Given the differences in the two assay platforms, we have demonstrated a potential mechanism by which the differences in the specificity of the two kits is mediated. The clonality of EIA antibodies in determining the specificity of assays has been previously described by other authors for other proteins [35,36]. Of course, other mechanisms may also be responsible for this difference, including the specificity of the primary antibody to different epitopes on the OXT molecule as illustrated by Gnanadesikan *et al.* (2022) [14]. It is worth noting however that for the vast majority of proteins this level of cross-reactivity was below the sensitivity and/or lower limit of detection of both assays, making this finding hard to interpret.

Relatively few studies have made head-to-head comparisons of the accuracies of different immunoassays for measuring plasma OXT, although none of these utilized human plasma [22,37,38]. Our findings further support the need for careful validation of commercially available EIAs prior to use in clinical research. In this study, the Cayman Chemical Company kit was selected for comparison mainly because of the significant differences in its format compared to other EIAs which were more similar to the Enzo Life Sciences kit (e.g. Arbor Assays, Michigan, USA; Abcam, Cambridge, UK). The Cayman Chemical Company kit demonstrated superior specificity and reliability, with measured unextracted plasma OXT concentrations intermediate between those previously reported for extracted vs. unextracted plasma using other kits (however note that even then the spike recovery for 200 pg/ml was just outside the reference range of 70–130% recommended by certain authors [39]). It is worth noting that although generally speaking reported extracted plasma OXT concentrations are 10- to 1000-fold lower than unextracted measurements, several studies are not consistent with this, either reporting unextracted concentrations of <10 pg/ml [40–42], or extracted concentrations of >100 pg/ml [10,31,33]. The superior specificity of the Cayman Chemical Company kit vs. the Enzo Life Sciences kit, particularly on unextracted samples, is in keeping with the findings of other authors who have found that the readings obtained from the Cayman Chemical Company kit with and without plasma extraction were similar [22].

More recently, mass spectrometry (MS) studies, widely regarded as the gold standard for biochemical analysis, have suggested that plasma protein binding may also play a significant role in measured OXT concentrations, with a pre-analysis reduction/alkylation step with DTT and iodoacetamide resulting in total OXT concentrations of 500–1900 pg/ml [6,17], in contrast to MS studies not incorporating this step (0-3.67-27 pg/ml) [18,43]. The clinical utility of measuring total vs. free plasma OXT concentrations remains unknown [1], and MS techniques remain subject to extraction processes. Further comparative studies are needed to examine the effects of reduction, alkylation and denaturation of proteins in unextracted plasma in immunoassays to determine their effects on plasma OXT measurements.

Further work still needs to be done to fully validate these assay techniques [38,44] and improve the quantification of plasma OXT concentrations, particularly given the widespread use of various unvalidated and potentially unreliable immunoassays and other analytical techniques in the explosion of OXT-related clinical research over the last decade. A recent review further showed that sex, age, time of day and smoking status all additionally affect plasma OXT concentrations [45]. Given these marked inconsistencies, it is therefore unsurprising that the reported physiological and neurobehavioral associations with plasma OXT concentrations are varied and often contradictory.

In this study, we only attempted to optimize a reversed phase SPE and an ultrafiltration technique for clinical use, but did not subject other SPE columns or plasma extraction techniques (e.g. liquid-liquid, ion exchange, gel filtration and high performance liquid chromatography) to the same validation processes using pure OXT solutions. We have also not manipulated other aspects of the SPE technique used here, such as using positive pressure. It is worth noting that for the Cayman Chemical Company kit the manufacturer recommends a reversed SPE process with methanol and acetone and therefore further work needs to be done to determine if this technique improves extraction efficiency. Only two previous studies have made head-to-head comparisons between various plasma purification techniques, both of which used spiked human plasma as a means of calculating recovery efficacy [8,23]. Further work also needs to be done to validate the use of pure OXT in different matrices apart from water, including assay buffers and phosphate-buffered saline (PBS).

We were unable to further confirm the specificity of immunoassays with the use of OXT-depleted human plasma which is not commercially available. Apart from Gnanadesikan *et al.* (2021) [22]; who used plasma from $Oxt^{-/-}$ knockout mice (there is no known equivalent OXT mutation in humans), only one other study has attempted to measure plasma OXT concentrations in individuals who are supposedly unable to produce OXT (anencephalic fetuses) [46]. However, this presumes that OXT production only occurs in the central nervous system – and there is evidence to suggest that other organs such as the pancreas and adrenal gland may also produce their own OXT [47,48]. With regards to determining the effects of OXT on neurobehavioral processes, cerebrospinal fluid OXT would be a better measure of its central activity, bearing in mind that the data on the correlation between cerebrospinal fluid and plasma concentrations is conflicting [9,11,13]. However, this is only obtainable by invasive techniques, which in themselves may cause changes in OXT concentrations given its reported association with stress [49]. We also did not perform studies in assay parallelism (due to the lack of native plasma with naturally high plasma OXT concentrations) and further work needs to be done to compare the concentrations obtained here with other techniques such as MS. Concentrating plasma samples to obtain higher plasma readings to allow for this risks the possibility of concurrently increasing the degree of interference.

Although we did briefly examine the efficacy of aprotinin on a single plasma sample in inhibiting the degradation of plasma OXT, we did not perform further direct comparisons with other protease inhibitors. Aprotinin is a serine protease inhibitor, inhibiting the activity of trypsin, chymotrypsin, kallikrein and plasmin [50–52]. Contrastingly, OXT is classically degraded by oxytocinase, a zinc-dependent leucine/cysteine aminopeptidase, but outside of pregnancy this is largely membrane bound [4,53]. Previous studies have shown that aprotinin does not prevent OXT degradation in the first 60 min and it has no activity against leucine aminopeptidases [5,54]. However, given that OXT contains amino acid residues such as glycine and tyrosine, which can be targeted by serine proteases it is reasonable to postulate that OXT is subject to degradation by other proteases as well. The subsequent stability of plasma OXT concentrations in this study for up to 10 days could be explained by the use of EDTA-containing collection tubes, as EDTA is a broad-spectrum inhibitor of metalloproteinases [55]. One previous study has suggested that OXT degradation is best inhibited by a combination of 1 M EDTA and 0.125 M phenanthroline, but also reported that the latter could interfere with immunoassays [5]. Further work therefore needs to be done to clarify the mechanism of OXT degradation and consequently determine the best means of maintaining the stability of OXT in plasma for analysis. It must also be noted that our experiments did not examine the efficacy of aprotinin on frozen samples which is the common means by which plasma is stored in the medium-term prior to analysis for OXT.

To conclude, we have here determined the internal reliability and low cross-reactivity of a monoclonal secondary antibody-based EIA for analysing unextracted plasma OXT concentrations. Our findings cast doubt on the common presumption that plasma extraction results in more accurate measurements of plasma OXT, as well as on the reported clinical associations with OXT in studies that have not clearly performed internal validation of their quantification techniques. Additionally, we have highlighted the urgent need to establish more robust methods of quantifying plasma OXT, particularly with newer biochemical methods such as MS.

Author contributions

H.W.G. and C.L. designed and performed the experiments in this study. H.A. and M.T.D. provided laboratory support and technical advice for the overall conduct of the study. All authors reviewed the manuscript prior to submission.

Role of the funding source

This work was supported by the BUPA Foundation (grant code 1DAAG), Great Ormond Street Hospital Children's Charity (1DAAJ), the National Institute for Health Research Great Ormond Street Hospital Biomedical Research Centre (1DAAN), and the British Society for Paediatric Endocrinology and Diabetes (1DAAP). None of these funders were involved in the study design, data collection or interpretation, or publication of this manuscript.

Declaration of competing interest

None.

Acknowledgments

The authors would like to thank the volunteers, and the laboratory staff in the department of chemical pathology at Great Ormond Street Hospital for Children NHS Foundation Trust and the Genetics & Genomic Medicine Research and Training Department at the University College London Great Ormond Street Institute of Child Health for their technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cpnec.2023.100188.

References

[1] E.A. Lawson, The effects of oxytocin on eating behaviour and metabolism in humans, Nat. Rev. Endocrinol. 13 (2017) 700–709, https://doi.org/10.1038/nrendo.2017.115.

[2] E.L. MacLean, S.R. Wilson, W.L. Martin, J.M. Davis, H.P. Nazarloo, C.S. Carter, Challenges for measuring oxytocin: the blind men and the elephant? Psychoneuroendocrinology 107 (2019) 225–231, https://doi.org/10.1016/j.psyneuen.2019.05.018.

[3] B.A. Tabak, G. Leng, A. Szeto, K.J. Parker, J.G. Verbalis, T.E. Ziegler, M.R. Lee, I. D. Neumann, A.J. Mendez, Advances in human oxytocin measurement: challenges and proposed solutions, Mol. Psychiatr. 28 (2023) 127–140, https://doi.org/10.1038/s41380-022-01719-z.

[4] M. Tsujimoto, A. Hattori, The oxytocinase subfamily of M1 aminopeptidases, Biochim. Biophys. Acta 1751 (2005) 9–18, https://doi.org/10.1016/j.bbapap.2004.09.011.

[5] J.M. Burd, J. Davison, D.R. Weightman, P.H. Baylis, Evaluation of enzyme inhibitors of pregnancy associated oxytocinase: application to the measurement of plasma immunoreactive oxytocin during human labour, Acta Endocrinol. 114 (1987) 458–464.

[6] O.K. Brandtzaeg, E. Johnsen, H. Roberg-Larsen, K.F. Seip, E.L. MacLean, L. R. Gesquiere, S. Leknes, E. Lundanes, S.R. Wilson, Proteomics tools reveal startlingly high amounts of oxytocin in plasma and serum, Sci. Rep. 6 (2016), 31693, https://doi.org/10.1038/srep31693.

[7] M.E. McCullough, P.S. Churchland, A.J. Mendez, Problems with measuring peripheral oxytocin: can the data on oxytocin and human behavior be trusted? Neurosci. Biobehav. Rev. 37 (2013) 1485–1492, https://doi.org/10.1016/j.neubiorev.2013.04.018.

[8] A. Szeto, P.M. McCabe, D.A. Nation, B.A. Tabak, M.A. Rossetti, M.E. McCullough, N. Schneiderman, A.J. Mendez, Evaluation of enzyme immunoassay and radioimmunoassay methods for the measurement of plasma oxytocin, Psychosom. Med. 73 (2011) 393–400, https://doi.org/10.1097/PSY.0b013e31821df0c2.

[9] D.S. Carson, S.W. Berquist, T.H. Trujillo, J.P. Garner, S.L. Hannah, S.A. Hyde, R. D. Sumiyoshi, L.P. Jackson, J.K. Moss, M.C. Strehlow, S.H. Cheshier, S. Partap, A. Y. Hardan, K.J. Parker, Cerebrospinal fluid and plasma oxytocin concentrations are positively correlated and negatively predict anxiety in children, Mol. Psychiatr. 20 (2015) 1085–1090, https://doi.org/10.1038/mp.2014.132.

[10] R. Feldman, I. Gordon, O. Zagoory-Sharon, Maternal and paternal plasma, salivary, and urinary oxytocin and parent-infant synchrony: considering stress and affiliation components of human bonding, Dev. Sci. 14 (2011) 752–761, https://doi.org/10.1111/j.1467-7687.2010.01021.x.

[11] S.M. Kagerbauer, J. Martin, T. Schuster, M. Blobner, E.F. Kochs, R. Landgraf, Plasma oxytocin and vasopressin do not predict neuropeptide concentrations in human cerebrospinal fluid, J. Neuroendocrinol. 25 (2013) 668–673, https://doi.org/10.1111/jne.12038.

[12] E.A. Lawson, D.A. Marengi, R.L. DeSanti, T.M. Holmes, D.A. Schoenfeld, C. J. Tolley, Oxytocin reduces caloric intake in men, Obesity 23 (2015) 950–956, https://doi.org/10.1002/oby.21069.

[13] J. Martin, S.M. Kagerbauer, T. Schuster, M. Blobner, E.F. Kochs, R. Landgraf, Vasopressin and oxytocin in CSF and plasma of patients with aneurysmal subarachnoid haemorrhage, Neuropeptides 48 (2014) 91–96, https://doi.org/10.1016/j.npep.2013.12.004.

[14] G.E. Gnanadesikan, E.A.D. Hammock, S.R. Tecot, R.J. Lewis, R. Hart, C.S. Carter, E. L. MacLean, What are oxytocin assays measuring? Epitope mapping, metabolites, and comparisons of wildtype & knockout mouse urine, Psychoneuroendocrinology 143 (2022), 105827, https://doi.org/10.1016/j.psyneuen.2022.105827.

[15] J.C. Christensen, P.A. Shiyanov, J.R. Estepp, J.J. Schlager, Lack of association between human plasma oxytocin and interpersonal trust in a Prisoner's Dilemma paradigm, PLoS One 9 (2014), e116172, https://doi.org/10.1371/journal.pone.0116172.

[16] E. Johnsen, S. Leknes, S.R. Wilson, E. Lundanes, Liquid chromatography-mass spectrometry platform for both small neurotransmitters and neuropeptides in blood, with automatic and robust solid phase extraction, Sci. Rep. 5 (2015) 9308, https://doi.org/10.1038/srep09308.

[17] R.D. Semba, P. Zhang, M. Zhu, E. Fabbri, M. Gonzalez-Freire, R. Moaddel, M. Geng-Spyropoulos, L. Ferrucci, A targeted proteomic assay for the measurement of plasma proteoforms related to human aging phenotypes, Proteomics 17 (2017), https://doi.org/10.1002/pmic.201600232.

[18] G. Zhang, Y. Zhang, D.M. Fast, Z. Lin, R. Steenwyk, Ultra sensitive quantitation of endogenous oxytocin in rat and human plasma using a two-dimensional liquid chromatography-tandem mass spectrometry assay, Anal. Biochem. 416 (2011) 45–52, https://doi.org/10.1016/j.ab.2011.04.041.

[19] C. Pequeux, J.C. Hendrick, M.T. Hagelstein, V. Geenen, J.J. Legros, Novel plasma extraction procedure and development of a specific enzyme-immunoassay of oxytocin: application to clinical and biological investigations of small cell carcinoma of the lung, Scand. J. Clin. Lab. Invest. 61 (2001) 407–415.

[20] C. Bienboire-Frosini, C. Chabaud, A. Cozzi, E. Codecasa, P. Pageat, Validation of a commercially available enzyme ImmunoAssay for the determination of oxytocin in

plasma samples from seven domestic animal species, Front. Neurosci. 11 (2017) 524, https://doi.org/10.3389/fnins.2017.00524.

[21] M.Y. Dawood, F. Fuchs, Maternal and fetal oxytocin levels at parturition in a paraplegic woman, Eur. J. Obstet. Gynecol. Reprod. Biol. 12 (1981) 1–6.

[22] G.E. Gnanadesikan, E.A.D. Hammock, S.R. Tecot, C. Sue Carter, E.L. MacLean, Specificity of plasma oxytocin immunoassays: a comparison of commercial assays and sample preparation techniques using oxytocin knockout and wildtype mice, Psychoneuroendocrinology 132 (2021), 105368, https://doi.org/10.1016/j.psyneuen.2021.105368.

[23] D.R. Cool, D. DeBrosse, Extraction of oxytocin and arginine-vasopressin from serum and plasma for radioimmunoassay and surface-enhanced laser desorption-ionization time-of-flight mass spectrometry, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 792 (2003) 375–380.

[24] A.S. McNeilly, I.C. Robinson, M.J. Houston, P.W. Howie, Release of oxytocin and prolactin in response to suckling, Br. Med. J. 286 (1983) 257–259.

[25] T. Padayachi, R.J. Norman, K. Dhavaraj, M. Kemp, S.M. Joubert, Serial oxytocin levels in amniotic fluid and maternal plasma during normal and induced labour, Br. J. Obstet. Gynaecol. 95 (1988) 888–893.

[26] U. Nilsson, Soothing music can increase oxytocin levels during bed rest after open-heart surgery: a randomised control trial, J. Clin. Nurs. 18 (2009) 2153–2161, https://doi.org/10.1111/j.1365-2702.2008.02718.x.

[27] P. Chiodera, F. Louis, J.J. Legros, Simultaneous radioimmunoassay for plasma arginine-vasopressin and oxytocin using DEAE Sephadex A 25 extraction, J. Endocrinol. Invest. 7 (1984) 287–293, https://doi.org/10.1007/BF03351004.

[28] P.B. Gray, J.C. Parkin, M.E. Samms-Vaughan, Hormonal correlates of human paternal interactions: a hospital-based investigation in urban Jamaica, Horm. Behav. 52 (2007) 499–507, https://doi.org/10.1016/j.yhbeh.2007.07.005.

[29] A.M. Daubenbuchel, A. Hoffmann, M. Eveslage, J. Ozyurt, K. Lohle, J. Reichel, C. M. Thiel, H. Martens, V. Geenen, H.L. Muller, Oxytocin in survivors of childhood-onset craniopharyngioma, Endocrine 54 (2016) 524–531, https://doi.org/10.1007/s12020-016-1084-5.

[30] G. Gimpl, F. Fahrenholz, The oxytocin receptor system: structure, function, and regulation, Physiol. Rev. 81 (2001) 629–683.

[31] A. Elmadih, M.W. Wan, D. Downey, R. Elliott, J.E. Swain, K.M. Abel, Natural variation in maternal sensitivity is reflected in maternal brain responses to infant stimuli, Behav. Neurosci. 130 (2016) 500–510, https://doi.org/10.1037/bne0000161.

[32] S. Haraya, K. Karasawa, Y. Sano, K. Ozawa, N. Kato, H. Arakawa, Development of a highly specific enzyme immunoassay for oxytocin and its use in plasma samples, Ann. Clin. Biochem. 54 (2017) 101–106, https://doi.org/10.1177/0004563216645122.

[33] T. Sasaki, K. Hashimoto, Y. Oda, T. Ishima, M. Yakita, T. Kurata, M. Kunou, J. Takahashi, Y. Kamata, A. Kimura, T. Niitsu, H. Komatsu, T. Hasegawa, A. Shiina, T. Hashimoto, N. Kanahara, E. Shimizu, M. Iyo, Increased serum levels of oxytocin in "treatment resistant depression in adolescents (TRDIA)" group, PLoS One 11 (2016), e0160767, https://doi.org/10.1371/journal.pone.0160767.

[34] B. Loun, K.R. Copeland, F.A. Sedor, Ultrafiltration discrepancies in recovery of myoglobin from urine, Clin. Chem. 42 (1996) 965–969.

[35] M.R. Denburg, A.N. Hoofnagle, S. Sayed, J. Gupta, I.H. de Boer, L.J. Appel, R. Durazo-Arvizu, K. Whitehead, H.I. Feldman, M.B. Leonard, Chronic Renal Insufficiency Cohort study, investigators, Comparison of two ELISA methods and mass spectrometry for measurement of vitamin D-binding protein: implications for the assessment of bioavailable vitamin D concentrations across genotypes, J. Bone Miner. Res. 31 (2016) 1128–1136, https://doi.org/10.1002/jbmr.2829.

[36] Y. Nasser, R. Labetoulle, I. Harzallah, A.E. Berger, X. Roblin, S. Paul, Comparison of point-of-care and classical immunoassays for the monitoring infliximab and antibodies against infliximab in IBD, Dig. Dis. Sci. 63 (2018) 2714–2721, https://doi.org/10.1007/s10620-018-5144-y.

[37] E.L. MacLean, L.R. Gesquiere, N. Gee, K. Levy, W.L. Martin, C.S. Carter, Validation of salivary oxytocin and vasopressin as biomarkers in domestic dogs, J. Neurosci. Methods 293 (2018) 67–76, https://doi.org/10.1016/j.jneumeth.2017.08.033.

[38] G. Wirobski, F.S. Schaebs, F. Range, S. Marshall-Pescini, T. Deschner, Analytical and physiological validation of an enzyme immunoassay to measure oxytocin in dog, wolf, and human urine samples, Sci. Rep. 11 (2021), 12793, https://doi.org/10.1038/s41598-021-92356-z.

[39] M.-A. Valentin, S. Ma, A. Zhao, F. Legay, A. Avrameas, Validation of immunoassay for protein biomarkers: bioanalytical study plan implementation to support pre-clinical and clinical studies, J. Pharm. Biomed. Anal. 55 (2011) 869–877, https://doi.org/10.1016/j.jpba.2011.03.033.

[40] S. Caruso, D. Mauro, G. Scalia, C.I. Palermo, A.M.C. Rapisarda, A. Cianci, Oxytocin plasma levels in orgasmic and anorgasmic women, Gynecol. Endocrinol. 34 (2018) 69–72, https://doi.org/10.1080/09513590.2017.1336219.

[41] A. Ebert, M.A. Edel, P. Gilbert, M. Brune, Endogenous oxytocin is associated with the experience of compassion and recalled upbringing in Borderline Personality Disorder, Depress. Anxiety 35 (2018) 50–57, https://doi.org/10.1002/da.22683.

[42] G. Yuan, W. Qian, R. Pan, J. Jia, D. Jiang, Q. Yang, S. Wang, Y. Liu, S. Yu, H. Hu, W. Sun, J. Ye, C. Mao, R. Zhuang, L. Zhou, Reduced circulating oxytocin and High-Molecular-Weight adiponectin are risk factors for metabolic syndrome, Endocr. J. 63 (2016) 655–662, https://doi.org/10.1507/endocrj.EJ16-0078.

[43] A.A. Franke, X. Li, A. Menden, M.R. Lee, J.F. Lai, Oxytocin analysis from human serum, urine, and saliva by orbitrap LCMS, Drug Test. Anal. 11 (2019) 119–128, https://doi.org/10.1002/dta.2475.

[44] U. Andreasson, A. Petter-Lliaudet, L.J.C. van Waalwijk van Doorn, K. Blennow, D. Chiasserini, S. Engelborghs, T. Fladby, S. Genc, N. Kruse, H. Bea Kuiperij, L. Kulic, P. Lewczuk, B. Mollenhauer, B. Mroczko, L. Parnetti, E. Vanmechelen, M. M. Verbeek, B. Winblad, H. Zetterberg, M. Koel-Simmelink, C.E. Teunissen, A practical guide to immunoassay method validation, Front. Neurol. 6 (2015) 179, https://doi.org/10.3389/fneur.2015.00179.

[45] S. Engel, S. Laufer, R. Miller, H. Niemeye, C. Knaevelsrud, S. Schumacher, Demographic, sampling- and assay-related counfounders of endogenous oxytocin concentrations: a systematic review and meta-analysis, Front. Neuroendocrinol. 54 (2019), 100775, https://doi.org/10.1016/j.yfrne.2019.100775.

[46] Y. Otsuki, O. Tanizawa, K. Yamaji, M. Fujita, K. Kurachi, Feto-maternal plasma oxytocin levels in normal and anencephalic pregnancies, Acta Obstet. Gynecol. Scand. 62 (1983) 235–237.

[47] J.A. Amico, F.M. Finn, J. Haldar, Oxytocin and vasopressin are present in human and rat pancreas, Am. J. Med. Sci. 296 (1988) 303–307.

[48] V.T. Ang, J.S. Jenkins, Neurohypophysial hormones in the adrenal medulla, J. Clin. Endocrinol. Metab. 58 (1984) 688–691, https://doi.org/10.1210/jcem-58-4-688.

[49] V. Engert, A.M. Koester, A. Riepenhausen, T. Singer, Boosting recovery rather than buffering reactivity: higher stress-induced oxytocin secretion is associated with increased cortisol reactivity and faster vagal recovery after acute psychosocial stress, Psychoneuroendocrinology 74 (2016) 111–120, https://doi.org/10.1016/j.psyneuen.2016.08.029.

[50] C.J. Amris, Inhibition of fibrinolytic and thromboplastic activity by Trasylol, Scand. J. Haematol. 3 (1966) 19–32.

[51] A. Morgan, L.A. Robinson, T.T. White, Postoperative changes in the trypsin inhibitor activities of human pancreatic juice and the influence of infusion of Trasylol on the inhibitor activity, Am. J. Surg. 115 (1968) 131–139.

[52] H. Nagaoka, M. Katori, Inhibition of kinin formation by a kallikrein inhibitor during extracorporeal circulation in open-heart surgery, Circulation 52 (1975) 325–332.

[53] T. Rogi, M. Tsujimoto, H. Nakazato, S. Mizutani, Y. Tomoda, Human placental leucine aminopeptidase/oxytocinase. A new member of type II membrane-spanning zinc metallopeptidase family, J. Biol. Chem. 271 (1996) 56–61, https://doi.org/10.1074/jbc.271.1.56.

[54] A. Josephrajkumar, R. Chakrabarty, G. Thomas, Midgut proteases of the cardamom shoot and capsule borer Conogethes punctiferalis (Lepidoptera: Pyralidae) and their interaction with aprotinin, Bull. Entomol. Res. 96 (2006) 91–98.

[55] K. Lalu, S. Lampelo, T. Vanha-Perttula, Characterization of three aminopeptidases purified from maternal serum, Biochim. Biophys. Acta 873 (1986) 190–197.