REVIEW ARTICLE

Measuring Oxytocin and Vasopressin: Bioassays, Immunoassays and Random Numbers

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Journal of Neuroendocrinology

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In this review, we consider the ways in which vasopressin and oxytocin have been measured since their first discovery. Two different ways of measuring oxytocin in widespread use currently give values in human plasma that differ by two orders of magnitude, and the values measured by these two methods in the same samples show no correlation. The notion that we should accept this seems absurd. *Either* one (or both) methods is not measuring oxytocin, *or*, by 'oxytocin', the scientists that use these different methods mean something very different. If these communities are to talk to each other, it is important to validate one method and invalidate the other, or else to establish exactly what each community understands by 'oxytocin'. A similar issue concerns vasopressin: again, different ways of measuring vasopressin give values in human plasma that differ by two orders of magnitude, and it appears that the same explanation for discrepant oxytocin measurements applies to discrepant vasopressin measurements. The first assays for oxytocin and vasopressin measured biological activity directly. When immunoassays were introduced, they encountered problems: high molecular weight factors in raw plasma interfered with the binding of antibodies to the hormones, leading to high and erroneous readings. When these interfering factors were removed by extraction of plasma samples, immunoassays gave measurements consistent with bioassays, with measures of turnover and with the sensitivity of target tissues to exogenous hormone. However, many recent papers use an enzyme-linked immunoassay to measure plasma levels without extracting the samples. Like the first radioimmunassays of unextracted plasma, this generates impossibly high and wholly erroneous measurements.

Introduction

Vasopressin and oxytocin are released into the circulation from the axon terminals of magnocellular hypothalamic neurones that project to the posterior pituitary. They are also released within the brain from the dendrites of these neurones, in a manner regulated semi-independently of axonal release. In addition, diverse populations of functionally separate neurones release oxytocin and vasopressin within the brain to regulate diverse physiological processes and behaviours (1). The involvement of these peptides in social behaviour has led some to measure them in plasma, with the assumption that these might be indicative of their activity in the brain. The sense behind this has been questioned (2). Furthermore, more than 100 papers have recently been published using assays that have been claimed to measure oxytocin and vasopressin in human plasma simply and reliably (for example 3–6), but which report levels massively inconsistent with previous assays. Here, we seek to describe and explain these inconsistencies.

A brief history

In 1906, as an aside in a study of the actions of ergot, Dale (7) reported that extracts of the posterior pituitary gland could stimulate uterine contractions. In 1909, Bell (8) conducted the first clinical trial of this extract, reporting that it powerfully contracted the uterus of women in labour. The extract swiftly entered use in obstetric practice and, in 1913, the Professor of Obstetrics and Gynecology at the University of Toronto declared that 'it has been employed in practically every obstetrical clinic throughout this continent and in Britain and Europe' (9).

In 1925, a Committee of the League of Nations adopted the United States Pharmacopeia Standard Reference Powder as an

international standard (10). This was made from the whole posterior lobes of cattle: the glands were collected immediately after death and ground in acetone to remove water and fat, with 0.5 mg of powder declared equivalent to one International Unit (IU). These extracts were standardised for their oxytocic activity by a bioassay (on guinea pig uterus), although it was clear that they contained at least two active principles: one with both a pressor activity and antidiuretic activity, and one with oxytocic activity (11). In 1928, Kamm (12) separated and concentrated the two principles, and named them 'vasopressin' and 'oxytocin'.

In the 1950s, the amino acid sequences and structures of oxytocin and vasopressin were elucidated, followed shortly by their synthesis. However, the units by which they were measured remained tied to biological activity. In 1955, the Third International Standard was established by an international collaboration: samples of posterior pituitary extract were sent to 19 laboratories in 10 countries, where 185 assays were carried out to define its oxytocic, vasopressor and antidiuretic potency (13). The standard was adjusted to have the same biological activity as previous standards, and its activity was compared with that of pure synthesised peptide. The current (4th) International Standard for Oxytocin assigns 1 mg of oxytocin a potency of 600 IU (14), and the International Standard for Vasopressin assigns 1 mg of vasopressin a potency of 410 IU (15), and these are the values used for conversions in the present review.

By the end of the 1950s, it was accepted that oxytocin and vasopressin were synthesised in hypothalamic neurones, packaged into vesicles in the cell body and transported down the axons for storage in the axon terminals in the pituitary (16). The vesicles certainly contained much more than the hormones: Gainer *et al.* (17,18) identified two putative precursor proteins in the hypothalamus, and showed that they were packaged into the neurosecretory vesicles where they were processed to produce the peptides and their associated neurophysins. The structures of the precursors were determined (19), and neurophysins, now established to be elements of the precursors, were thus revealed to be stored and secreted with their associated peptides in equimolar amounts.

Morris (20) then sought to establish exactly how much hormone is present in each vesicle. By counting vesicles in ultrathin sections of the pituitary under the electron microscope, he estimated that the rat posterior pituitary contains 1.44×10^{10} vesicles and, from the pituitary content of oxytocin and vasopressin measured by bioassays, he deduced that each vesicle contains approximately 84 000 hormone molecules. This calculation assumed that all of the hormone content was contained in the vesicles and so, with Jean Nordmann, he set about checking this. They prepared fractions of posterior pituitary in which the number of vesicles could be estimated, by adding latex particles of similar size, pelleting the mixture, and measuring the ratio of latex particles to vesicles (21). They measured hormone content by both radioimmunoassays and bioassays, and neurophysin content by radioimmunoassay, and concluded that vesicles from both oxen and rats contained approximately 85 000 molecules of hormone and neurophysin. The close agreement with the estimate derived by morphometry of the whole gland confirmed that all hormone was contained within the vesicles. The estimated number of molecules in each vesicle was close to the theoretical maximum in the space available, and so could not be a significant underestimate.

Oxytocin and vasopressin act at four receptors: the oxytocin receptor (22); the V2 receptor, which accounts for the antidiuretic actions of vasopressin (23,24); the V1b receptor (25), which accounts for vasopressin effects on adrenocorticotrophin release; and the V1a receptor, which accounts for the pressor activity of vasopressin (26). These receptors bind their preferred ligands with nanomolar affinity, although each hormone can act at receptors for the other if present at a 100-fold excess. Receptor affinity is important for comparing the potency of different peptides at a given target, although it is a poor guide to the concentrations needed to elicit biological activity. The effective concentration in any tissue depends less on the receptor affinity than on the receptor density. In the periphery, V2 receptors are at highest density in the kidney, and oxytocin receptors are at highest density in the uterus and mammary gland.

Bioassays of vasopressin

Initially, the main ways of quantifying oxytocin and vasopressin were by bioassays. The 'pressor' bioassay measured the blood pressure response to i.v. injection of extracts in rats (27). Specificity was a problem because other factors present in samples might also raise blood pressure. Accordingly, in the protocol of Dekanski (27), rats were injected with an α-adrenergic antagonist to abolish the pressor responses to adrenaline and noradrenaline, which were the major confounding factors.

Bioassays for antidiuretic activity measured the degree to which injections of extract concentrated the urine of a water-loaded rat (28), and these had fewer problems with specificity than pressor assays. By the late 1950s, antidiuretic assays could measure vasopressin at doses as low as 4 μU (10 pg) (29), a sensitivity achieved by injecting rats with alcohol to suppress endogenous vasopressin secretion and by maintaining a constant water load to stabilise the preparation for repeated measurements. These assays were still not sufficiently sensitive to measure in plasma until Heller and Stulc (30) introduced another refinement; using rats with exteriorised urinary bladders, they could detect concentrations above 0.65 μU/ml, and they estimated that vasopressin circulated in normally hydrated men at approximately 1.7 μU/ml (4 pg/ml).

Using a modified version of this assay, Czaczkes *et al.* (31) took blood samples from men and assayed them without extraction or concentration. Vasopressin was undetectable (< 0.25 μU/ml) in samples taken 1–2 h after an oral water load, but, during 24 h of dehydration, concentrations rose to approximately 18 μU/ml (44 pg/ml), whereas urine osmolarity increased from approximately 70 mOsm/l to more than 1000 mOsm/l. The range agreed with that reported by Heller and Stulc (30), and also with that reported by Yoshida *et al.* (32) who found 1.9 μU/ml (4.6 pg/ml) in extracted samples from normally hydrated subjects, rising to 6.5 μU/ml (16 pg/ml) after modest dehydration.

The antidiuretic bioassay was capable of high sensitivity and precision, and it yielded some remarkable insights. For example, Segar

and Moore (33) found that vasopressin concentrations in man depend on both position and on ambient temperature. In 79 control subjects who were sitting comfortably, the mean plasma concentration was 1.65 μU/ml (4 pg/ml) compared to 0.4 μU/ml (1 pg/ml) when lying down and 3.1 μU/ml (8 pg/ml) when standing. The responses to posture reflect the sensitivity of vasopressin secretion in man to central hypovolaemia, and were later confirmed by radioimmunoassay (34–36). After 2 h of exposure to 50 °C, levels in control subjects rose to 5.2 μU/ml (13 pg/ml), falling again within 15 min at 26 °C. The sensitivity to temperature was also later confirmed by a radioimmunoassay (37) and, in 2008, it was reported that vasopressin neurones are intrinsically thermosensitive through membrane channels expressed from the trpv1 gene (38).

From such studies came the agreement that the normal plasma concentration of vasopressin in man is below 2 μU/ml (5 pg/ml) (39), with this increasing several fold after 24 h of dehydration. These conclusions were also consistent with studies of antidiuretic responses to exogenous vasopressin: Theobald (40) first showed that injection of 5–10 mU (12–24 ng) vasopressin produced near-maximal antidiuresis in man (see also 41–43), and studies using i.v. infusions of vasopressin in water-loaded subjects indicated that the kidney responds to changes in the rate of vasopressin secretion of < 1 pg/min/kg, and also that the rate required to produce isosmolar urine during overhydration is approximately 3 pg/min/kg (44–46). A similar sensitivity to low levels of vasopressin was found in dogs (47,48).

Bioactivity of oxytocin

Antidiuretic assays benefitted from the extreme sensitivity of the urine concentrating mechanisms of the kidney, but practical bioassays for oxytocin were less sensitive. The uterus is most sensitive to oxytocin in late pregnancy (22) but, at this stage, the uterus contracts spontaneously, complicating the measurement of samples. Bioassays using the nonpregnant rat uterus (49) and mammary gland bioassays (42) could generally detect only concentrations above 25 μU/ml (42 pg/ml). The most sensitive bioassays used mouse mammary strips and could detect 4 μU/ml (7 pg/ml) in 0.5 ml of sample (50), although even this was still not sufficiently sensitive to measure levels in raw plasma.

The first estimates of the circulating concentration of oxytocin in man came not from any direct bioassay but were a combination of deduction and experiment (40,51). Theobald (51), who had shown that very low doses of vasopressin evoked antidiuresis in man, speculated that similarly low doses of oxytocin would induce labour, and he showed that drips delivering 1–5 mU/min (2–8 ng/min) were indeed effective. He concluded that the physiological concentration of oxytocin which initiates labour 'could not be more and might well be less' than that achieved by this rate of delivery. Saameli (52) drew similar conclusions: he calculated that the half-life of oxytocin in pregnant women was approximately 3–4 min, and also that uterine contractions were initiated by infusions that raised plasma concentrations by 3 μU/ml (5 pg/ml).

Although oxytocin infusions advance labour, and although there is a modest increase in plasma oxytocin concentrations before delivery in most animal studies, many have doubted whether there is *any* increase in oxytocin secretion before delivery in women (42,53–55). They have proposed that it is not an increase in oxytocin secretion, but the increase in sensitivity of the uterus to oxytocin that initiates labour in women (56). Nevertheless, augmenting oxytocin concentrations with infusions can advance delivery in all mammals studied. Most protocols for inducing labour in women start by infusing oxytocin at 1–2 mU/min (1.7–3.3 ng/min), rising to 20–30 mU/min for at most 5 h, with a maximum total dose of 5 IU (8 μg). Higher doses than these are not more effective (57), and some have suggested that even these rates are higher than necessary. Dawood (58) advised that infusions should not exceed 2–8 mU/min and reported that oxytocin given as pulses, beginning with 1 mU/min every 8 min and doubling the pulse dose every 24 min, was as efficient as continuous infusion. Oxytocin is given at much higher doses to prevent post-partum haemorrhage (59), but this action appears to be mediated not by oxytocin receptors but by V1a receptors on the uterine artery (60).

Theobald (51) also studied the effect of suckling during the first 10 days of the puerperium; uterine contractions began within 3 min of the onset of suckling, and this effect was matched by a single i.v. injection of 10 mU (17 ng) oxytocin. Other studies recorded intramammary pressure in women in the week after delivery, and noted that i.v. injections of just 0.1–2 mU (0.2–3 ng) caused sharp increases in intramammary pressure (61), and when a baby suckled at the uncannulated breast, sharp rises in intramammary pressure were seen in the cannulated gland, similar to those evoked by i.v. injection of approximately 5 mU of oxytocin (62,63). The human myopithelium proved to be more sensitive to oxytocin than in any other species studied previously. Measurements by radioimmunoassay reported that, during suckling in women, intermittent pulses were observed with a mean amplitude of just approximately 9 pg/ml from a basal level of approximately 2 pg/ml, as expected for bolus release of approximately 17 ng (64).

In lactating rats, suckling results in intermittent activation of oxytocin neurones: every 5–10 min, they discharge a burst of approximately 150 action potentials over 2–3 s. Each burst is followed by an increase in intramammary pressure that can be mimicked by i.v. injection of approximately 0.5 mU (0.8 ng) of oxytocin (16,65). In anaesthetised rats, similar increases in intramammary pressure can be produced by electrically stimulating the pituitary stalk for 4 s at 50 Hz to evoke a burst-like train of action potentials (65). Similar stimulation of the isolated posterior pituitary gland *in vitro* evokes secretion into the bathing medium, which can be measured by radioimmunoassay without problems of sample matrix interference because the assay standard curve is generated in the same medium. Stimulation at 50 Hz releases approximately 7 pg of oxytocin per stimulus pulse (66), which is close to the release estimated from the intramammary pressure response to a similar electrical stimulation *in vivo*.

The plasma volume of a rat is approximately 4 ml/100 g (67), and so a burst that releases 0.5 mU (0.8 ng) oxytocin should raise circulating concentrations in a 300 g rat by approximately 70 pg/ml.

Higuchi *et al.* (68) developed a radioimmunoassay that allowed oxytocin to be measured in unextracted rat plasma, and took samples at the time of suckling-induced milk let-down, as recognised by stretching movements of the suckling pups. Plasma concentrations rose from 16.5 pg/ml to approximately 65 pg/ml, and declined with a half-life of approximately 1.5 min. Because the behavioural response is apparent only with a delay after milk let-down, these samples may have missed the full peak of plasma oxytocin, although the agreement is still close.

In pigs (body weight 150–200 kg), suckling evokes increases in intramammary pressure equivalent to those induced by i.v. injection of 50 mU (83 ng) of oxytocin (69). For a plasma volume of 5 l, these would raise plasma concentrations by approximately 17 pg/ml, which is close to the peak concentration (approximately 18 pg/ml) measured by radioimmunoassay during suckling-induced milk ejection. Larger animals show even higher sensitivity to oxytocin; in horses (body weight 543 kg), with injections of just 5–10 mU mimic the suckling-induced increase in intramammary pressure (70).

Across mammalian species, blood volume is linearly proportional to body mass, although the mass of the pituitary is proportional only to the log of body mass (71). Consistent with this, the human posterior pituitary contains approximately 21 µg of oxytocin (72–74), whereas that of the rat contains 0.5–1 µg. Given a 400-fold difference in plasma volume but only a 40-fold difference in pituitary oxytocin content, it is likely that the higher sensitivity of the mammary gland and uterus in humans parallels the lower amounts of oxytocin available for secretion from the pituitary.

Immunoassays

By the end of the 20th century, bioassays were still 'of critical importance' in the discovery of novel antagonists and antagonists of vasopressin and oxytocin because biological activity cannot be inferred from structure alone (75). However, bioassays were time consuming and expensive in their use of animals, and they had been extensively replaced by immunoassays that could assay many samples quickly. In 1973, Chard (76) noted that best immunoassays for vasopressin were no more sensitive than the best bioassays; the same is still true today for most radioimmunoassays and most enzyme-linked immunoassays (ELISAs). However, bioassays are less specific because of the cross-reactivity of vasopressin and oxytocin with their respective receptors (76).

Radioimmunoassays and competitive ELISAs both depend on the reaction between the hormone in a sample and a fixed amount of antibody added to it: some of the hormone will bind to the antibody, whjereas some remains 'free', in a reaction governed by the law of mass action. When a fixed amount of radiolabelled or enzyme-conjugated hormone is added, it competes for binding to the antibody with the hormone already present, and so less labelled hormone will bind when more unlabelled hormone is present. In radioimmunoassays, the bound fraction is then precipitated, usually with a second antibody that recognises the original antibody. The amount of label in the pellet is measured, and the values are compared with a standard curve generated from known concentrations of unlabelled hormone in assay buffer (processed in the same way as the samples). This is a hyperbolic curve, which, after allowing for nonspecific binding, can be reduced by a logit-log transformation to a straight line (77). In ELISAs, the bound fraction is retained in the assay plate, and the amount of bound label is measured by optical density, giving a similar hyperbolic standard curve.

There were considerable difficulties in developing immunoassays for vasopressin and oxytocin. Because these have a low molecular weight, they are poor immunogens, making the preparation of high-affinity antisera difficult, and circulating levels were clearly very low. Thus, close attention was given to extraction and concentration procedures (76). In 1970, Robertson *et al.* (78) described one of the first radioimmunoassays to measure plasma vasopressin in man. This assay was sensitive to 1 pg/ml (0.3 mU/ml) but, when whole plasma was assayed, apparent concentrations were 'several hundredfold higher than anticipated, failed to fluctuate appropriately in response to physiological manipulation or in disease states, and were not reduced substantially by dialysis of the plasma under conditions that completely removed vasopressin'.

Robertson *et al.* (78) set about understanding why these initial radioimmunoassay measurements were erroneous. By filtering plasma samples on G-25 Sephadex, they resolved three peaks of 'immunoreactivity', only the third and smallest of which was consistent with true vasopressin, being recovered in the same location as pure vasopressin (78). The first peak accounted for a 67% loss of binding of antibody to vasopressin, and the second peak accounted for a further 30% loss. Neither of these apparent 'immunoreactivities' were displaced by an excess of cold vasopressin, indicating that they did not involve competitive binding of vasopressin, but occlusion of antibody recognition of vasopressin. The immunoreactivity in the third peak reproduced the expected relationship with plasma osmotic pressure in normal adults, the expected high levels in nephrogenic diabetes insipidus (in which the kidney is unresponsive to vasopressin, resulting in high plasma osmolarity and elevated vasopressin secretion) and the expected low levels in pituitary diabetes insipidus (in which vasopressin secretion is deficient as a result of genetic mutation or to damage to the hypothalamus or pituitary). After accounting for the plasma matrix interference, Robertson *et al.* (78) calculated that normal plasma concentrations of vasopressin in man were approximately 1 pg/ml, increasing to approximately 10 pg/ml with dehydration.

Problems with immunoassays

Problems can come from factors such as heparin or low pH or high osmolality that interfere with the antigen–antibody reaction. High salt concentrations have a nonlinear effect, and serial dilutions of samples with a high salt content produce measurements that do not parallel the standard curve. In some cases, an antibody might recognise elements unrelated to the antigen against which it was raised. Antibodies are sensitive to differences in amino acid sequence, although they recognise the structure of a molecule, not its amino acid sequence *per se*, and so unrelated molecules might cross-react (76,79). However, it is unlikely that an antibody will recognise a different ligand with higher affinity than it has for the

ligand to which it was raised; it may recognise other ligands with lower affinity, although this presents a major problem only if that different ligand is present at higher concentrations.

For peptide assays, interference mainly comes from plasma proteins that bind to the primary antibody, impairing its ability to bind hormone, leading to an excess of free label that is erroneously measured as a high concentration of hormone (78) (Fig. 1). Such interference has also been recognised in immunoassays for cholecystokinin (80), parathyroid hormone (81), thyrotropic hormone (82), glucagon (83), secretin (84,85), angiotensin (86), growth hormone (87), vasoactive intestinal peptide (88), gonadotrophins (89) and gonadotrophin-releasing hormone (90), all of which were resolved by sample extraction. When describing their vasopressin assay, Baylis and Heath (91) emphasised that 'even small increases in protein concentration lower binding and therefore careful control of extract protein concentration is needed to avoid the misinterpretation that a fall in binding is due to immunoreactive vasopressin'. At that time, seven other vasopressin radioimmunoassays had been described, using different antibodies and different extraction procedures. All had reported plasma concentrations of 1–10 pg/ml, and all showed that concentrations changed with physiological conditions in the manner expected from bioassays.

Fyhrquist et al. (92) circumvented the problem of protein interference by using individual plasma blanks spiked with a known amount of vasopressin to correct measured values. In unextracted plasma from healthy subjects, they found a mean vasopressin concentration of 2 pg/ml in the supine position and 6.2 pg/ml in the upright position, which is in agreement with the levels measured in bioassays by Segar and Moore (33). Henneberry et al. (93) took a different approach: they assayed unextracted samples using standard curves generated in charcoal-filtered plasma, and reported basal levels of 1.8 pg/ml, rising to 6.3 pg/ml after a saline infusion that raised plasma osmotic pressure from 280 to 293 mOsm/l.

Immunoassays for oxytocin

By the 1980s, many laboratories had generated radioimmunoassays for oxytocin, using independently produced antibodies (58,72–74,94–98)] and most, but not all (99,100), required extraction. With few exceptions, assays in extracted plasma reported basal levels of < 5 pg/ml in man: of 17 studies reviewed by Szeto et al. (101), only two reported higher levels; the highest (71 pg/ml) were in samples taken from the corpus cavernosum of the penis, a circumstance that might be expected to affect hormone secretion.

Antibodies do not recognise the whole molecule, and so an immunoassay may overestimate hormone concentrations by measuring fragments of no physiological significance (76). Generally, antibodies are raised to the tail end of oxytocin, which confers its biological activity. When oxytocin or vasopressin are secreted, the entire contents of the vesicle are discharged, including elements of incompletely processed precursor that may not be biologically active. If an extended or truncated oxytocin molecule has full biological activity, then, to all intents and purposes, it is oxytocin, as understood to be the active molecule secreted from the posterior pituitary. Problems might arise less from assays recognising such

forms, than from *failing* to recognise them, leading to an underestimate of the levels of bioactive oxytocin. However, the major discrepancies are that some immunoassays massively *overestimate* oxytocin concentrations by comparison with bioassays.

Overestimation might arise if an antibody recognises a fragment with higher affinity than the native peptide, although this is unlikely. Overestimation can also arise when enzymes are present that degrade oxytocin. The plasma of late pregnant women contains abundant oxytocinase, which inactivates oxytocin by splitting the peptide linkage between the cysteine and tyrosine residues (102). When oxytocin is incubated in this plasma, there is a rapid loss of biological activity but, after 2 h, oxytocin 'immunoreactivity' exceeds bioactivity by three-fold. The obvious explanation is that oxytocinase in the sample also degrades the labelled oxytocin. The consequence will be that less label will bind to antibody, leading to overestimation of the amount of oxytocin (76). Fortunately, enzymatic degradation of oxytocin is a problem only when measuring in plasma from pregnant women (54), and extraction procedures can remove such enzymes efficiently.

The relationship between plasma concentrations and rates of production and secretion

How much hormone is present in plasma depends on the rate at which it is secreted, the volume in which it is dispersed, and the rate at which it is cleared. Hormone is secreted into the extracellular fluid of the pituitary gland; from there, it enters blood that drains into the jugular vein and, unless it is bound to much larger molecules in the plasma, it will pass relatively freely between the plasma and the extravascular fluid. Thus, the 'distribution volume' will be between the total plasma volume and the total extracellular fluid volume. The clearance can be measured by the decline of hormone in blood samples taken after an i.v. injection or at the end of a steady-state infusion, usually by a half-life approximation to an exponential fit to the first, fast phase of disappearance. Other studies prefer to measure the metabolic clearance rate (MCR), by infusing hormone at a fixed rate until plasma concentrations reach an equilibrium. At equilibrium, the delivery of oxytocin must equal its clearance, and the MCR is calculated from the delivery rate and the measured concentration. The MCR is measured in units of l/min, being the equivalent volume of fluid from which hormone is completely cleared every 1 min.

Clearance of vasopressin

In dogs, Lauson and Bacanegra (103) reported that the antidiuretic activity of exogenous vasopressin disappeared with a half-life of approximately 5.4 min. In experiments on the isolated, perifused kidney, it was found that, when vasopressin was infused at 100–120 ng/min, approximately 38% of it was cleared in each passage through the kidney. More was lost from the blood than was found in the urine, and Lauson and Bacanegra (103) concluded that, at physiological levels (< 25 pg/ml), most of the vasopressin in the plasma is bound to protein and is cleared by the tubular cells of the kidney by enzymatic inactivation and secretion. At higher
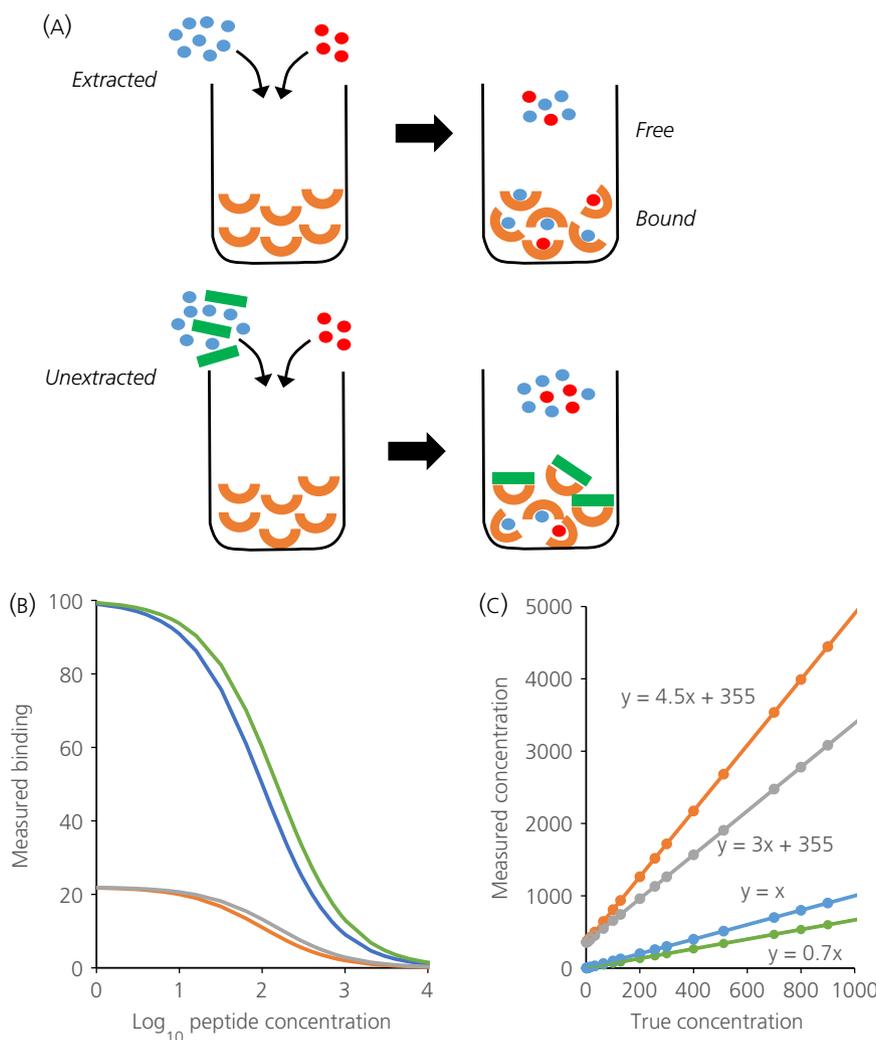
Fig. 1. (A) Illustrates the principles underlying competitive immunoassays and the problem posed by plasma matrix interference. In an immunoassay, a sample containing an unknown amount of hormone (in blue) is mixed with a known amount of labelled hormone (red) and introduced to a given amount of antibody (orange). The labelled and unlabelled hormone compete for binding to the antibody: the more hormone in the sample, the less bound labelled hormone, which is what is measured in an immunoassay. Unextracted plasma contains large molecules that can bind nonspecifically to antibodies (green), preventing them from binding to antigen. Accordingly, less labelled hormone can bind to the antibody, and this is erroneously measured as an excess of hormone in the sample. When a ligand L interacts with antibody M to form a complex ML, the concentrations at equilibrium are governed by $K_d = [M]*[L]/[ML]$ where K_d, the dissociation constant, characterises the affinity of the antibody. Defining $M_o = [M] + [ML]$ as the *maximum binding*, the relationship $[ML] = M_o*[L]/(K_d + [L])$ determines the hyperbolic standard curve (78). By this relationship, in a radioimmunoassay, the log of the concentration of unlabelled peptide is linearly proportional to logit (B/B_o) where B is the counts measured $([M])$, B_0 is the maximum number of counts measured in the absence of unlabelled peptide $([M_0])$, and logit $(y) = \log_e(y/(1 - y))$. (B) Illustrating (in blue) the relationship between log peptide concentration and B when B_0 and K_d are given values of 100. Samples are contained not in assay buffer but in a sample matrix that can affect either B_o or K_d. If the matrix reduces the affinity of the antibody, the true concentrations of ligand will relate to measured levels by a curve that is displaced to the right of the standard curve: the green curve in (B) simulates the effect of changing K_d from 100 to 150. In an immunoassay, this leads to an underestimation of levels, as shown by the green line in (C), which plots the true concentration against that inferred from the standard curve. If the matrix reduces the antibody available for binding with the ligand, the true concentrations will relate to measured levels by a curve that is 'compressed'. The orange curve in (B) simulates the effect of interference that changes B_o from 100 to 22; such an assay will report an erroneous high basal level (360 pg/ml) and will exaggerate the levels of any ligand present, by 4.5-fold in the example shown by the green line in (C). Plasma proteins may affect both K_d and B_o. The grey curves in (B) and (C) show the effects of increasing K_d to 150 and reducing B_0 to 22. The erroneous baseline level is unaffected, but the exaggeration is reduced; here, the gain is three-fold.

concentrations, more of the vasopressin is unbound, and some of this is filtered at the glomerulus and excreted into urine.

In rats, Ginsburg and Heller (104) estimated that the initial half-life of exogenously administered vasopressin was less than 1 min.

From experiments in acutely nephrectomised rats, and by ligating arteries to reduce blood flow through the liver, it was concluded that most of the vasopressin was cleared by the kidneys and liver. In similar studies on rabbits, vasopressin was cleared with a

half-life of approximately 4.5 min, again mainly by the kidneys and liver (105). In dogs, rather than measure vasopressin by either radioimmunoassay or bioassay, Ang and Jenkins (106) injected radiolabelled vasopressin and used thin-layer chromatography and autoradiography to quantify labelled vasopressin in plasma samples: it disappeared with an initial half-life of 3 min and a later half-life of 13 min.

In man, after an acute injection of vasopressin, bioactivity disappeared with an initial half-life of 5.6 min (43). Later studies using radioimmunoassay indicated that vasopressin disappeared from the circulation with a bi-exponential decay (107). The fast phase had a half-life of 5.4 min and a distribution volume close to the plasma volume; the slower phase had a half-life of 78 min with a distribution volume close to the extracellular fluid volume. In patients with chronic renal failure, the clearance is much slower: MCR is reduced by approximately half (108), which is consistent with the conclusion that vasopressin is mainly cleared from the circulation through the kidneys.

Clearance of oxytocin

Ginsburg and Smith (49), using a rat uterus bioassay, found that, when oxytocin was added to rat plasma *in vitro* at 37 °C, it was relatively stable, losing just 6–13% of biological activity after 40 min of incubation. By contrast, when oxytocin was injected into rats, the biological activity disappeared with a half-life of 1.65 min. In nephrectomised rats, bioactivity declined with a half-life of approximately 3 min in the first 7 min, and much more slowly thereafter. When the splanchnic vessels were also ligated, the initial decline was not further changed but, after 7 min, oxytocin concentrations 'stuck' at a high, steady level. Ginsburg and Smith deduced that, when oxytocin enters the circulation, it is rapidly removed by the kidneys and liver. Without these organs, injected oxytocin diffuses within approximately 7 min throughout the extracellular fluid, where it is degraded only very slowly.

In the baboon, oxytocin clearance was also consistent with a two-compartmental system, corresponding to the plasma volume and the extravascular fluid volume. The initial half-life in the first phase of distribution was approximately 1 min, followed by a terminal elimination half-life of approximately 10 min (109).

In man, Fabian *et al.* (43) measured oxytocin with a mammary gland bioassay and studied its clearance after the end of an i.v. infusion. Bioactivity disappeared with a half-life of 4.8 min with a distribution volume of 10.5 ml/100 g, which is close to the extracellular fluid volume, whereas an acute large injection disappeared with a half-life of 3.2 min and a distribution volume of 7.4 ml/100 g. Ultrafiltration of the plasma indicated that, whereas approximately 30% of similarly infused vasopressin was bound to large molecules, there was no apparent binding of oxytocin. *In vitro*, oxytocin bioactivity was stable, as found in rats (49); only approximately 20% was lost after 24 h at 37 °C.

Thornton *et al.* (110) measured oxytocin by radioimmunoassay in extracted plasma from pregnant and post-partum women, taking careful precautions to inactivate oxytocinase. Within 30 min, infusions of 17.9 ng/min in pregnant women raised oxytocin concentrations by 3.5 pg/ml from a basal level of 1.5 pg/ml. In postpartum women, the same increase could be matched by infusion of just 4.3 ng/min. The corresponding MCRs were 5.7 l/min in pregnant women but only 1.3 l/min in postpartum women, suggesting that oxytocinase indeed has a major effect on the clearance of oxytocin.

Thus, bioassays, radioimmunoassays and evidence of the sensitivity of peripheral targets to oxytocin and vasopressin all converged on the conclusion that, in mammals, both hormones are normally present in the circulation at < 10 pg/ml. The pharmokinetics are consistent with a two-compartment model, where these hormones are secreted into the blood are cleared by the kidneys and liver, and diffuse between the blood and extravascular fluid. Bolus injections disappear rapidly, with an initial half-life that varies between species and with physiological state: in the rat, both hormones disappear with an initial half-life of approximately 2 min; in man, oxytocin has a half-life of 4–10 min, except in pregnancy, when oxytocinase shortens this to 2–3 min. As a proportion of body weight, the rat pituitary contains much more oxytocin and vasopressin than the human pituitary and, in rats, as in other small mammals, circulating levels are higher. In man and other large animals, lower circulating concentrations are compensated for by higher sensitivity of the peripheral targets.

ELISAs

Competitive ELISAs work on the same principles as radioimmunoassays, with the advantages that they do not use radioactive materials, require less investment in equipment, and the biotinyl–oxytocin is more stable than radiolabelled oxytocin. However, ELISAs are similarly susceptible to plasma interference. Discussing the problems that arise in measurements in human plasma, Bjerner *et al.* (111) characterised the first step of any immunoassay as 'the encounter between a limited number of immobilised animal antibodies in close relation to each other and a huge concentration of human immunoglobulins and non-immunoglobulins containing a broad spectrum of reactivities'. The binding of plasma proteins to an immunoglobulin can hinder its ability to bind an antigen, reducing its affinity, and/or may completely prevent some of the antibody from binding to antigen. With a reduction in maximum binding, an assay will report erroneously high values even in the absence of any antigen, and will exaggerate measurements of added antigen. The effect of a reduction in affinity is different; in this case, the assay will under-report added antigen. In both cases, there will be a linear relationship between the amount of oxytocin added and the amount measured, although the slope will depend on the nature of the interference (Fig. 1).

In 1994, the first ELISA suitable for measuring oxytocin in plasma was developed by Prakash *et al.* (112). They recognised that plasma components interfered by reducing antibody binding, and so they constructed standard curves in hormone-stripped plasma. Their assay could detect plasma concentrations above 1.25 pg/ml, and they used it to measure oxytocin in the cow during milking, comparing the results with measurements of the same samples by radioimmunoassay after plasma extraction. The two methods showed very close agreement.

The ELISAs in most widespread use are marketed by Enzo, who draw attention to problems of plasma matrix interference, illustrate the benefits of plasma extraction, and 'strongly recommend' that users extract plasma samples. They state that it might be possible to dilute plasma samples enough to minimise interference (suggesting at least an eight-fold dilution) but warn that the user 'must verify' that these dilutions are appropriate for their samples (113). This advice has been largely ignored. Many studies have used this assay on unextracted human plasma, reporting mean concentrations of oxytocin and vasopressin of typically 100–500 pg/ml (3–6,114). Those that have diluted samples have done so by less than the manufacturer's recommendations, without confirming that their dilution is adequate.

These values are inconsistent, by two orders of magnitude, with both bioassays of oxytocin and vasopressin and with radioimmunoassay measurements on extracted samples. They are also inconsistent with the known sensitivity of peripheral targets to oxytocin and vasopressin, and with what is known of the production, storage and clearance of these hormones. A plasma concentration of 350 pg/ml oxytocin in man implies a total blood content of approximately 1 μg (given a plasma volume of 3 l) and, for a half-life of 10 min, this implies a secretion (and production) rate of 500 ng/min, or 720 IU/day. Jones and Pickering (115) measured the rate of hormone production in the rat by giving rats an intracisternal injection of tritiated tyrosine to label newly synthesised oxytocin and vasopressin. Radioactive hormone began to appear in the posterior pituitary within 2 h, and, after 7 days, the specific radioactivity of each hormone declined with a half-life of approximately 13 days. They thus calculated that oxytocin must be secreted at 18.7 mU/day and vasopressin at 28.9 mU/day. Assuming a half-life of 2 min, this is consistent with plasma concentrations of 5–10 pg/ml for each hormone. Thus, to sustain a plasma concentration of 350 pg/ml in man, oxytocin must be produced at 40 000 *times* the rate measured in the rat. Given a difference in pituitary mass and oxytocin content of just 40-fold, this seems beyond plausibility.

Three studies have compared measurements of oxytocin in the same samples by the same ELISA, with and without extraction (101,116,117). Robinson *et al.* (116) measured oxytocin levels in seals: in extracted samples, they found plasma levels of approximately 8 pg/ml, compared to 300 pg/ml in unextracted samples collected in heparin vacutainers or 540 pg/ml in unextracted samples collected in ethylenediaminetetraacetic acid (vacutainers). There was no correlation between the measurements in extracted and unextracted samples. Christensen *et al.* (117) measured plasma samples using both a radioimmunoassay and the Enzo ELISA. Radioimmunoassay of extracted samples returned mean levels of 1.1 pg/ml, and levels measured in the same samples spiked with oxytocin were consistently higher by the expected amount. Without sample extraction, the ELISA measured basal levels of 97 pg/ml, failed to recover spiked levels accurately, and the measurements did not correlate with levels measured in extracted samples. Szeto *et al.* (101) also found no correlation between levels in extracted and unextracted samples. Spiking unextracted samples with oxytocin resulted in a 3.5-fold exaggeration of the measurements,

indicating that plasma factors interfere with the binding of antibody to oxytocin (Fig. 1).

Claims that the Enzo ELISA is a valid way of measuring oxytocin in unextracted plasma do not rest on manufacturer's advice, which, as we have noted, strongly recommends extraction, but, instead, converge on two papers, neither of which actually investigated human plasma at all. Kramer *et al.* (118) measured oxytocin in plasma of rats and prairie voles, reporting basal levels of 79 pg/ml in rats and 264 pg/ml in voles, which are both much higher than measured by radioimmunoassay in extracted samples. They showed that pooled samples from prairie voles diluted in parallel with the assay standard curve. However, nonspecific binding to antibody is also governed by the law of mass action and, accordingly, erroneous levels can dilute in parallel to the standard curve except at extreme dilutions: what constitutes a sufficient dilution will depend on the affinity of the particular interfering factor for the antibody. Kramer *et al.* (118) also found that, in voles injected s.c. with 5 μg of oxytocin, plasma levels reached 20 ng/ml. Oxytocin injected s.c. is expected to distribute throughout the extracellular fluid volume, which, for a vole weighing approximately 70 g is < 10 ml. An injection of 5 μg should therefore increase plasma concentrations by > 500 ng/ml, much more than reported, suggesting that vole plasma proteins also reduce the affinity of the antibody.

Carter *et al.* (119) further reported that, in voles, the ELISA measured spiked levels of oxytocin with a gain of 0.79. This is consistent with a reduction in antibody affinity, although not sufficient to account for the discrepancy between expected and measured oxytocin in Kramer's measurements. In fractions separated by high-performance liquid chromatography (HPLC), apparent immunoreactivity appeared only in fractions that eluted with oxytocin, suggesting that there was no plasma interference. However, the same method had previously been used by Wismer Fries *et al.* (120) in a study that reported levels of oxytocin in human urine approximately one million times higher than found in previous or subsequent reports. According to Anderson (121), that discrepancy arose from the limited analytical selectivity of rapid chromatographic separation and the low sensitivity of ultraviolet absorbance detection.

Carter *et al.* (119) also claimed that four previous studies had validated the ELISA in unextracted plasma. Of those four studies, three neither report, nor cite *any* validation of the assay; the fourth (122) found higher plasma concentrations of oxytocin in rats than previously reported, and so conducted 'a control study with known concentrations of oxytocin'. No details are provided except that the ELISA kit 'had a detection sensitivity of 3.21 pg/ml'. Because they had found basal levels of 400 pg/ml in unextracted plasma, it appears that their control must simply have been a verification of assay performance in assay buffer.

Finally, Carter *et al.* (119) claimed a biological validation for the ELISA in reporting that breastfeeding in women *lowered* salivary concentrations of oxytocin. Because suckling-induced oxytocin release is indispensable for milk let-down in mammals (123), it is difficult to understand how such an anomalous observation qualifies as a validation.

Other methods of measurement

Attempts have been made to develop assays using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Such methods also have problems with sample matrix interference (124). As Zhang et al. (125) recognised, 'One common challenge in biomarker analysis is to select an appropriate surrogate matrix to prepare the standard curve, which is free of target analytes and is identical or similar to unknown sample matrix with regard to ionisation effects and extraction recovery'. Plasma oxytocin was measured using human plasma diluted with water (1 : 6) as a surrogate matrix, and basal levels of 2.4 pg/ml for human but 298 pg/ml for rat were reported; these high levels in the rat may indicate that human plasma is not a suitable surrogate matrix for measurements in rat. A similar LC-MS/MS assay was used to measure vasopressin in human plasma, again using human plasma diluted with water (1 : 6) as a surrogate matrix, and this reported levels < 2 pg/ml in children (126). Tsukazaki et al. (127) developed an LC-MS/MS assay for vasopressin, and showed that, correcting for plasma matrix effects, this assay gave close quantitative agreement with radioimmunoassay results from extracted human plasma samples, and had higher sensitivity and needed smaller sample volumes. So far, however, LC-MS/MS assays have not been developed to become a practicable alternative for general use.

Recently, many studies have measured copeptin in plasma as a surrogate marker for vasopressin (128). Copeptin is a large glycoprotein product of the vasopressin precursor, and its large size (37 kDa) makes it much easier to measure. As a fragment of the vasopressin precursor, it is released in equimolar amounts as vasopressin: it has a similar half-life to vasopressin in normal individuals and, in plasma, it circulates at approximately equimolar concentrations as the vasopressin that is measured by radioimmunoassay in extracted samples (129). No comparable surrogate marker for oxytocin has yet been identified.

Recommendations for validation of assays

Plasma matrix proteins interact differently with different antibodies: there is no single 'radioimmunoassay' and no single 'ELISA', and their susceptibility to plasma interference will vary with the antibody. Plasma proteins differ from individual to individual and differ substantially from species to species, and so any assay in unextracted plasma should be validated for the species in which it is measured and, ideally, in the individual in which it is measured.

It is natural to expect that measured levels should be consistent with measures of bioactivity. For vasopressin in man, testing this is straightforward. After a gastric water load, antidiuretic activity is minimal, as is evident from the production of highly dilute urine; conversely, high antidiuretic activity is evident after a modest period of dehydration from the production of highly concentrated urine. Thus, samples in these two conditions should show the extremes of very low and near maximal physiological levels. From bioassays and well-validated radioimmunoassays, this range is from 1 to approximately 10 pg/ml in man. In mice or rats, validation

could be supported by measurements in gene knockout animals or hypophysectomised animals, where vasopressin and/or oxytocin are completely absent.

There are no known conditions in which oxytocin is absent or grossly elevated in man. However, given the sensitivity of peripheral targets in man to very low concentrations, levels exceeding 10 pg/ml should be treated with scepticism. Levels from an individual could be verified by constructing a standard curve in hormone-stripped plasma from that individual (93), although it would be impractical to do this for every individual sampled.

Whatever assay is employed, consideration must be given as to how the sample matrix affects it. Analytical verification by HPLC with chromatographic separation (78) can confirm that measurements reflect the actual peptide, although care is needed to ensure that all high molecular weight proteins are eluted from the column and are checked for assay interference. There should be quantitative agreement between the amount of hormone in the appropriate fraction, and the level reported by the assay.

Samples with high levels will not dilute in parallel to the standard curve if the levels arise from factors such as high salt levels or pH that interfere nonlinearly with antibody binding, although this test may not recognise protein interference that blocks antibody recognition of the antigen. Such interference will be apparent if, when samples are spiked with known amounts of hormone, the assay recovers amounts different to the expected amount. However, the absence of a difference is not conclusive because interference that reduces antibody affinity will lead to underestimation of added hormone, whereas interference that blocks antibody recognition will lead to overestimation, and both effects are likely to be present.

Measuring oxytocin or vasopressin in cerebrospinal fluid, urine or saliva should have fewer problems because of the relative absence of proteins but, in urine, high salt concentrations may cause problems. In a steady-state, unbound oxytocin and vasopressin should distribute in extravascular fluids, and measurements in saliva might be useful for estimating steady-state secretion (119,130,131). However, matrix interference can be a problem in measurements in saliva at least for some antibodies, again because of nonspecific interference with antibody–antigen binding (132).

Conclusions

The high levels of oxytocin and vasopressin reported by immunoassays in unextracted plasma probably arise from plasma protein interference with antibody binding that produces a high and wholly erroneous baseline. If so, these assays are, for unextracted plasma, no more than a random number generator, returning arbitrary values with a high variance and a skewed distribution. Nevertheless, this extensive literature is not without interest. If our analysis is correct, then this literature becomes a test of the conclusion of Ioannidis (133) that most published results are false, arising from a combination of underpowered studies, confirmation bias and publication bias, where apparent statistical significance is generated by 'undisclosed researcher degrees of freedom' (134). Indeed, none of the four studies cited in the Introduction (3–6) as examples of the use of ELISAs to measure oxytocin and vasopressin in unextracted

samples made any correction for multiple comparisons in their analyses.

The current impetus for measuring vasopressin and oxytocin in plasma derives from the assumption that such measurements are informative of the release of these peptides in the brain. The fallacies in this supposition have been discussed elsewhere (2). A recent systematic review has concluded that studies using ELISA on unextracted samples have produced no convincing evidence that peripheral vasopressin or oxytocin might be reliable biomarkers in psychiatric disorders (135). It would be a pity if studies based on unsound presumptions and erroneous measurements are taken as evidence that disturbances in peptide release within the brain are not involved in these conditions.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

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