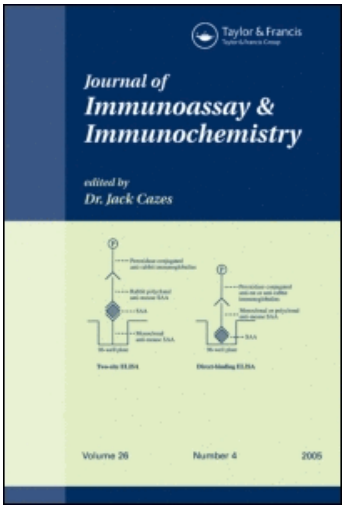

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Solid Phase Radioimmunoassay for Direct Measurement of Human Plasma Oxytocin

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SOLID PHASE RADIOIMMUNOASSAY FOR DIRECT MEASUREMENT OF HUMAN PLASMA OXYTOCIN

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ABSTRACT

Synthetic oxytocin (OT) conjugated to bovine thyroglobulin by the carbodiimide reaction was injected into rabbits to raise a high titre, specific OT antiserum which was then coupled to microcrystalline cellulose activated by cyanogen bromide. High affinity of the coupled antiserum was defined by Scatchard analysis, Keq = 7.1 x 10^{11} l/mol. Cross-reactivity studies revealed little binding of antiserum to analogues of OT. Iodination was performed by the Chloramine T method, giving specific activity of ^{125}I-OT, range 1.1 - 1.7 x 10^{3} Ci/mmol. After incubation for 40 hours under disequilibrium conditions, specific and non-specific bindings were 10.6 ± 2.7% and 0.2 ± 0.1% (n=15), respectively. Displacement of 50% ^{125}I-OT occurred with 2.9 pg OT/tube. Coefficients of variation of standard OT concentrations (0.03 - 16 pg/tube) were < 5%. Limit of detection was 2 pg OT/ml plasma. Recovery of synthetic OT added to non-pregnant plasma was 81.8% (n=34) at 20 pg/ml and 97.4% (n=32) at 100 pg/ml. Two patients, 17 and 18 weeks post-partum, had increases in plasma OT from < 2 pg/ml to 18.3 and 16.0 pg/ml after 6 and 4 minutes breast feeding infants, respectively. We conclude that this solid phase OT radioimmunoassay is quick, relatively sensitive and reliable, and does not require prior extraction of plasma samples.

INTRODUCTION

Oxytocin is the neurohypophysial hormone commonly thought to play a major role in the control of lactation and contraction of the uterus during pregnancy. At these times it is present in plasma in large quantities, but basal circulating levels are

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very low, in the order 1.5 to 4 pg/ml as measured by Leake et al [1], Weitzman et al [2], Kumaresan [3] and Amico [4]. With the exception of Kumaresan, these workers as well as many others have used extraction techniques prior to assay of oxytocin from plasma. These methods are required to eliminate nonspecific interference factors in the plasma and also to concentrate the oxytocin, but inherent in all such techniques which are, in general, technically demanding and time consuming, is the reduced recovery. While Dogterom et al [5] claimed 55% recovery of oxytocin with Vicor Glass, and Weitzman reported 73% efficiency with acetone, [2], Dawood [6] only recovered 42% using Fuller's earth. In an attempt to overcome these problem areas, we have developed a solid phase radioimmunoassay for OT that is capable of detecting basal circulating levels but does not require prior extraction of the plasma sample.

MATERIALS AND METHODS

Synthetic oxytocin (OT) for use in raising antisera and iodination was obtained from Ferring AB, who also donated the analogues tocinoic acid, dNH_2 - Tyr (OEt) - OT, lysine vasopressin, arginine vasotocin and arginine vasopressin. Oxytocin used for standards in the assay was the 4th International Standard (76/575) obtained from the National Institute for Biological Standards and Control, London, UK. The analogues isotocin, mesotocin, pro-leu-gly and oxytocin (free acid) were obtained from BaChem (UK) Ltd. All other chemicals were Analar grade unless indicated otherwise.

Antisera to oxytocin were raised by conjugation of oxytocin to bovine thyroglobulin (Sigma, UK, Ltd) with carbodiimide (Sigma, UK, Ltd) in the ratio 118:1:200, OT:BTG:CDI as described by Skowsky and Fisher [7]. The reagents were mixed at 20°C for 17 hours; then an equal volume of 1 mol/l amonium hydroxide, pH 6.6, was added and mixing continued for a further 2 hours. The conjugate was purified on Sephadex G25F (Pharmacia) in 0.02 mol/l phosphate buffer, pH 7.2, and then emulsified with an equal volume of Freuds adjuvant (Difco Laboratories, UK) and given as multiple site intradermal injections at 3-4 month intervals over a period of 12 months into white New Zealand rabbits. Scatchard analysis was carried out on data from standard dose-response curves.

Iodination was performed by a modification of the Chloramine T method described by Greenwood and Hunter [8]. The reaction mixture was purified on a Sephadex G25F column, eluting with 0.25% acetic acid buffer containing 1.25 mg/ml bovine serum albumin, fraction V (Sigma, UK, Ltd). Specific activity was calculated by diluting ^{125}I-OT parallel to standard dose-response curves and relating quantity of iodinated OT to standard synthetic OT. Efficiency of gamma counter was 70%. Binding of iodination peaks to excess OT antiserum was assessed, and TCA precipitation studies were also performed on peaks.

Microcrystalline cellulose for thin layer chromatography (Merck, F.R.G.) was activated by cyanogen bromide using a modification of the method of Wide [9]. Cyanogen bromide was dissolved in cold distilled water at 25 mg/ml and an equal amount of cel-

lulose added, stirring continuously. The pH was kept at 10-11 by titration with 1 mol/l NaOH for 2 minutes. The suspension was filtered through a G5 Sinta Funnel and washed well with copious quantities of cold distilled water, followed by increasing concentrations of cold aqueous acetone until neat acetone was used. The polymer was pulled dry for at least 2 hours and then stored in sealed vials at -20°C, where it remained stable for at least 1 year. Activated cellulose (100 mg) was mixed with 25 µl neat antiserum and 400 µl 0.1 mol/l sodium bicarbonate by continuous rotation at 20°C for 18 hours. Thereafter the immunosorbent was washed as described previously [10].

Assay buffer used throughout was 50 mmol/l Tris-HCl, pH 7.5, with bovine serum albumin (3.5 mg/ml) and 0.02% sodium azide as preservative. Standard curves were obtained using 500 µl of immunosorbent at 50 µg/ml assay buffer together with 200 µl standard serialy diluted from 80 pg/ml to 0.15 pg/ml. Buffer replaced the standard in zero (initial) binding tubes and non-specific binding tubes contained 500 µl cellulose at 50 µg/ml that had been washed identically to the immunosorbent. Tubes were capped and incubated at 20°C for 20 hours with continuous mixing. Thereafter 100 µl ^{125}I-OT (5000 cpm) was added and mixing continued for a further 20 hours before washing off the immunosorbent as described previously [10]. A number of analogues of OT and commonly occurring hormones were tested for their cross-reactivity by replacing the standard with 200 µl analogue or hormone. The amount of analogue required to displace 50% ^{125}I-OT is expressed

as a percentage of the amount of OT required to produce similar displacement.

Standard curves were also constructed containing 100 µl fasting plasma, in which immunosorbent was added as 400 µl at 62.5 µg/ml. In both forms of standard curve, the ratio bound ^{125}I-OT to total ^{125}I-OT was calculated for each standard and expressed as a percentage of zero binding to produce the standard curve. Recovery from unextracted male or non-pregnant female plasma was assessed by adding OT in the range 20 pg/ml to 100 pg/ml, and was calculated from a standard curve containing plasma (100 µl).

To investigate whether large molecular weight interfering substances were present plasma containing 1 ng synthetic oxytocin and trace amounts of ^{125}I-OT was chromatographed on a Sephadex G25 column, bed volume 200 ml. Fractions (4 ml) were collected, brought to pH 7.0-7.5 and 200 µl fractions were assayed directly.

Physiological validation of the assay was established by assaying directly oxytocin released during breast feeding. Lactating mothers were 17 and 18 weeks post-partum, a time when oxytocinase is no longer present. Observations were made at 10.00 am feeds. The infants were taken from the mothers at 9.30 am to prevent any anticipatory release of OT and kept in a separate room, whilst venous cannulae were inserted into the maternal forearms and resting 2 ml blood samples taken 15 minutes before suckling. Further blood samples were taken as suckling commenced and every two minutes thereafter for 10 minutes. Blood was drawn into chilled heparinised syringes and transferred to lithium heparin tubes kept

on ice. Two further samples were taken, at 5 and 15 minutes after suckling. Samples were centrifuged immediately at 4°C and the plasma kept on ice until assayed the same day.

RESULTS

Only one of six rabbits produced a suitable antiserum, binding 50% of ^{125}I-OT at a titre of 1 in 4.4 x 10^4. Iodination of oxytocin yielded two peaks after fractionation (Fig. 1). The first peak corresponded to the mono-iodinated ligand and gave a specific activity of 1.5 x 10^3 Ci/mmol, which is similar to the theoretical specific activity of mono-iodinated OT (1.8 x 10^3 Ci/mmol). The second peak gave a specific activity of 2.2 x 10^3 Ci/mmol and probably corresponded to the diiodo-ligand. Binding to excess OT antiserum was maximal with first peak. Mono-iodinated OT remained stable for up to 2 months, and was used as tracer in the assay.

Figure 2 shows the dose response-curve for the immunosorbent, mean ± 1 SEM (n=30). The smallest amount of oxytocin that could be detected, defined at 10% displacement of ^{125}I-OT was 0.2 pg/tube. Non-specific binding was 0.2 ± 0.1% while specific (zero) binding in the presence of antiserum only was 11.8% ± 1.8%. Coefficient of variation at each point on the standard curve (n=8) is also shown, and ranged from 2% to 5% (Fig. 2). Interassay coefficient of variation at 10 pg/tube was 11% (n=7). Scatchard analysis of the binding of OT to antiserum under equilibrium conditions revealed a two component antiserum, one with a high avidity component and low binding capacity, Keq = 7.1 x 10^{11} l/mol, and the other with a lower avidity, Keq = 7.2 x 10^{10} l/mol (Fig. 3).

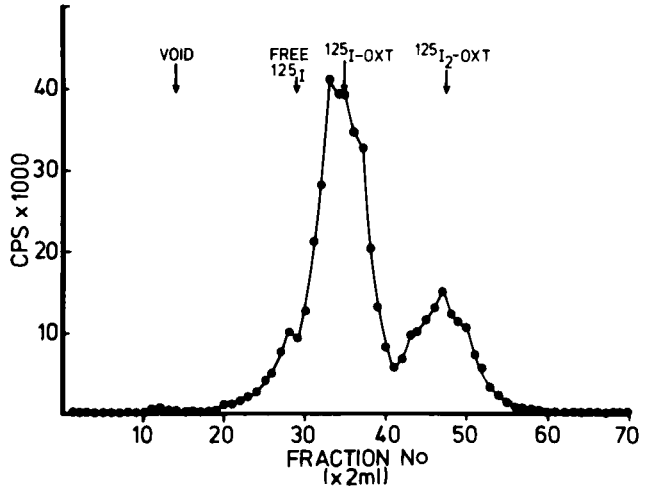

Figure 1 Elution profile of iodination of synthetic OT using chloramine T. Eluant was 0.25% acetic acid containing 1.25 mg/ml BSA and 0.02% sodium azide.

The antiserum is highly specific for OT and does not cross react appreciably with the neurohypophysial hormones, arginine vasopressin (AVP), lysine vasopressin (LVP) and arginine vasotocin (AVT). Relative displacements were:- OT, 100%; AVP, 0.10%; LVP, 0.22%; AVT, 0.14%. Cross reactivity-curves are shown in Figure 4, while analogues of OT gave the displacements presented in Table 1. None of the commonly occurring hormones including growth hormone, prolactin, ACTH, catecholamines, steroids, calcitonin, parathyroid hormone or thyroid hormone (at physiological levels) showed any cross reactivity with the antiserum.

Figure 5 shows the mean standard curve (n=15) obtained when plasma instead of buffer was added to the assay tubes compared to

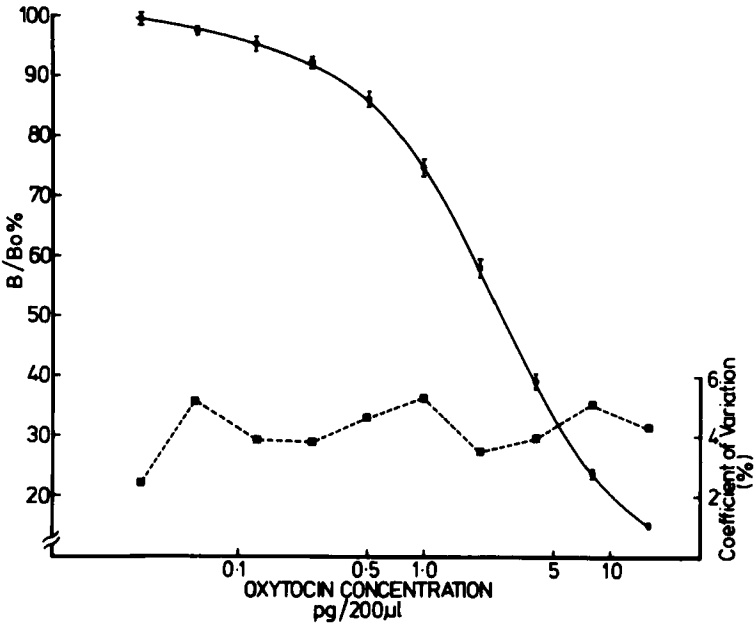

Figure 2 Dose response curve using immunosorbent at 50 µg/ml. Each point represents mean ± s.e.m. (n=30) specific binding was 11.8 ± 1.8% s.d. and non-specific binding was 0.2 ± 0.1%. Coefficient of variation at each point (n=8) was 2% to 5%.

the aqueous standard curve, and dilution of plasma with added synthetic OT. Immunosorbent concentration was increased to 400 µl at 62.5 µg/ml, which gave an identical concentration to that used in standard curves without plasma. Specific binding was 10.6 ± 2.7% and non-specific binding in tubes containing plasma remained at 0.2% ± 0.1% (n=15). Displacement of 50% ^{125}I-OT occurred at 2.9 pg OT/tube. The two standard curves were parallel, as was the curve produced by serial dilution of a sample of plasma containing 10 pg/ml oxytocin.

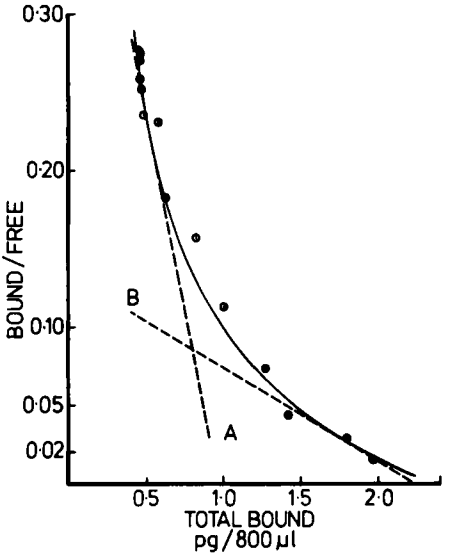

Figure 3 Scatchard analysis of binding of OT to the immunosorbent at equilibrium. Slope A Keq = 7.1×10^{11} l/mol and slope B Keq = 7.2×10^{10} l/mol.

Chromatography of a 2 ml aliquot of plasma containing 1 ng synthetic OT and a small amount of ^{125}I-OT produced a peak of immunoreactive oxytocin between fractions 17 and 21, corresponding to the peak produced when synthetic OT alone was chromatographed on the same column. Plasma eluted in the void volume (fractions 10-13) showed no OT immunoreactivity.

The mean recovery of synthetic oxytocin added to plasma at two concentrations was 81.8% at 20 pg/ml (n=34) and 97.4% at 100 pg/ml (n=32). The correlation coefficient between added and recovered oxytocin was +0.93. Plasma OT could be stored at -20°C for up to 3 months without loss of immunoreactivity. Intra- and interassay

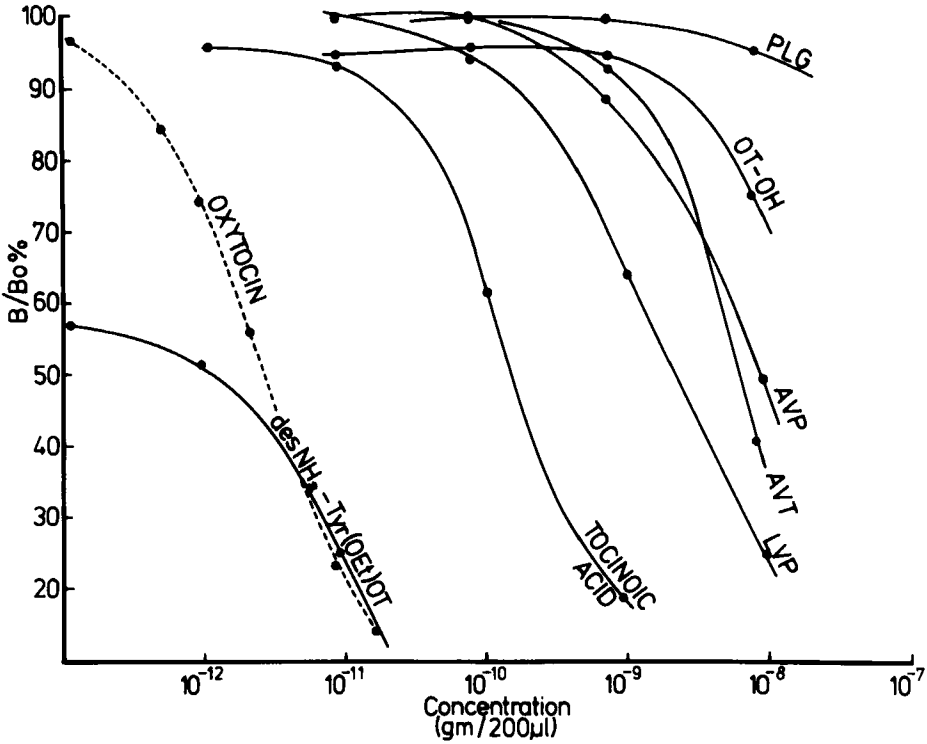

Figure 4 Cross reactivity studies of the immunosorbent with related peptides and analogues. PLG = ProLeuGly, OT-OH = Oxytocin, the free acid.

coefficients of variation at 100 pg/ml were 16.8 and 22.8% respectively (n=12).

Measurement of immunoreactive endogenous oxytocin from two breast-feeding mothers showed, in both cases, a sharp rise of plasma OT (Fig. 6) 2 minutes after the start of suckling with a peak of 18.3 pg/ml for JW after 6 minutes followed by a gradual decline to undetectable levels, the limit of detection of the

TABLE 1

Results of cross-reactivity studies showing the 50% displacements of analogues relative to OT.

Analogue	M.W.	Structure	Relative Molar Displacement (%)
Oxytocin	1007	Cys-Tyr-Ileu-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 (S — S)	100
Tocinoic Acid	741	- - -	2
Mesotocin	1007	Ileu	2.1
Isotocin	966	Ser Ileu	1.3
Oxytocin (free acid)	1008	-OH	< 0.07
PLG	285	- - - - - -	< 0.02
Arginine Vasopressin	1084	Phe Arg	0.10
Lysine Vasopressin	1024	Phe Lys	0.22
Arginine Vasotocin	1050	Ileu Arg	0.14
desNH_2 Tyr(OEt)OT	1036	(dNH_2) (OEt)	515

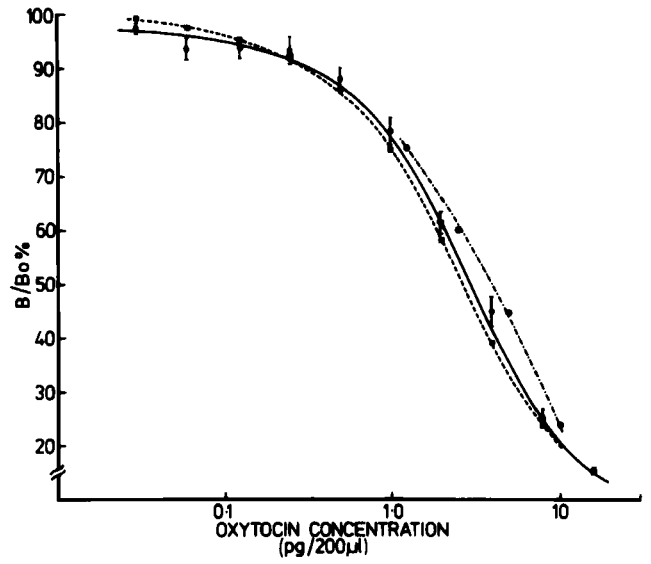

Figure 5 Dose response curves in the presence (● —— ●) and absence (■ --- ■) of 100 µl plasma. Results are mean ± s.e.m. (n=15). Specific binding in the presence of plasma was 10.6 ± 2.7% s.d. and non-specific binding 0.2 ± 0.1%. Dilution of plasma (o -•-•-•o) ran parallel to the standard curve.

assay being 2 pg/ml. SR reached a first peak after 4 minutes at 8.0pg/ml after which time the baby became restless and she changed breasts. Feeding from the second breast gave a peak of 16 pg/ml, after 4 minutes suckling.

DISCUSSION

A sensitive radioimmunoassay is dependent on production of a highly specific and high affinity antiserum with a stable radiolabelled tracer of adequate specific activity. We have raised an

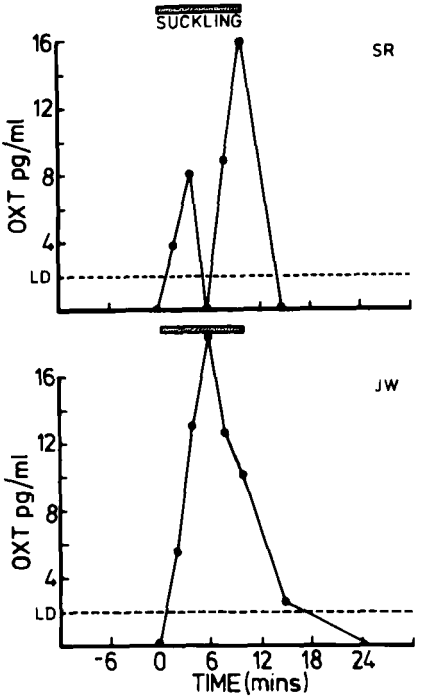

Figure 6 Plasma oxytocin levels in two cases of established lactation in response to suckling. Limit of detection (LD) was 2 pg/ml.

antiserum to OT that is highly specific for the nonapeptides, allowing it to distinguish OT from other naturally occurring nonapeptides and being unaffected by circulating levels of many hormones, notably those present during pregnancy, labour and lactation, the times when OT is reported to be most physiologically active. Cross reactivity studies to determine to which part of the molecule the antiserum binds indicate that both the tripeptide tail and the ring structure of OT are required for maximal binding.

Iodination of OT using chloramine T followed by purification on a Sephadex G25F column produces a satisfactory and stable tracer of high activity, close to the theoretical specific activity of mono-iodinated OT (1.8×10^3 Ci/mmol).

The use of antisera chemically coupled to an insoluble polysaccharide meet the criteria proposed by Ratcliffe [11] that are required for good separation technique. Firstly, complete separation of bound and free fractions is accomplished by the repeated washing of the bound pellet which removes all interfering factors. Secondly, the assay is relatively quick and simple to perform. The cellulose is kept in constant suspension allowing equal aliquots of antiserum to be added to each tube. Finally, the assay must not be affected by non-specific binding of the label and cause problems of standardisation, especially in double antibody precipitations. The use of an immunosorbent reduces binding of damaged labelled hormone to a minimum and eliminates serum or plasma factors by the repeated washing of the bound pellet, and it should be emphasized that this OT assay does not suffer from plasma interference. Solid phase assays are generally very precise, giving low coefficients of variation at all points on the standard curve, a fact confirmed by our precision profile which has coefficients of variation all less than 5%. There are two incubation steps; the initial preincubation is 20 hours and this, coupled with the addition of large amounts of tracer for the second incubation period (20 h) act to increase the sensitivity of the assay.

As this assay is sensitive and is unaffected by plasma factors, it is possible to use it to measure oxytocin directly in plasma

and eliminate extraction procedures. There are many techniques described by other workers to extract and concentrate OT from plasma. Dawood et al [6] used activated Fuller's earth, Leake [1] acetone/petroleum ether, Dogterom [5] Vicor Glass and McNeilly et al [12] in his study on breast feeding used Sep-Paks. Only three other authors have described assays which do not require prior extraction of plasma Kumaresan [3] measured plasma OT levels during parturition, in an assay that took 6 days to incubate. Prion-Bossuyt [13] reported work on OT in human umbilical cord plasma, and Robinson [14] has produced a sensitive and quick OT assay for use directly on dog and guinea pig plasma and CSF, but not human plasma. We have been able to measure OT directly in the plasma of breast feeding women, finding levels of OT comparable with those reported by other workers. Leake, in her study of OT release during breast pump stimulation obtained maximum levels of only 6.7 pg/ml in women 10 days to 3 months post-partum. McNeilly [12] found maximum concentrations ranging from 11 to 59 pg/ml, the latter being an anticipatory response which we excluded in our study by removal of the baby to another room. Drewett et al [15] found similar results in his study, as did Weitzman et al [2], but Dawood et al [16] found a peak plasma OT after 10 minutes of 53 pg/ml but started with a basal concentration of 10.8 pg/ml, while we, in agreement with other authors, found basal plasma OT levels to be less than 2 pg/ml.

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