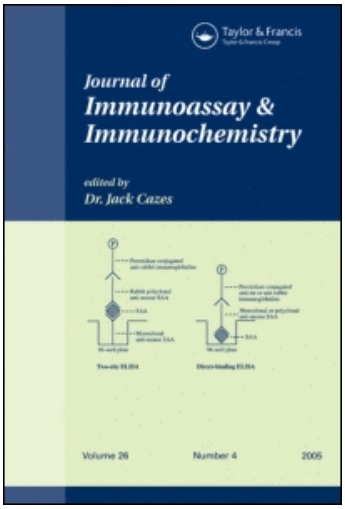

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Subpicogram Determination of Oxytocin by an Enzyme Immunoassay Using Acetylcholinesterase as Label

P. G. Marnet[a]; H. Volland[a]; P. Pradelles[a]; J. Grassi[b]; M. Beaufils[b]
[a] INRA - Laboratoire de Recherches sur la Traite ENSAR, Rennes, Cédex [b] CEA - Service de Pharmacologie et Immunologie CEA/CEN SACLAYF, Gif-sur-Yvette, Cédex

To cite this Article Marnet, P. G. , Volland, H. , Pradelles, P. , Grassi, J. and Beaufils, M.(1994) 'Subpicogram Determination of Oxytocin by an Enzyme Immunoassay Using Acetylcholinesterase as Label', Journal of Immunoassay and Immunochemistry, 15: 1, 35 — 53
To link to this Article: DOI: 10.1080/15321819408009570
URL: http://dx.doi.org/10.1080/15321819408009570

JOURNAL OF IMMUNOASSAY, 15(1), 35-53 (1994)

SUBPICOGRAM DETERMINATION OF OXYTOCIN BY AN ENZYME IMMUNOASSAY USING ACETYLCHOLINESTERASE AS LABEL

P.G. Marnet* , H. Volland*, P. Pradelles**, J. Grassi**, M. Beaufils*
*INRA - Laboratoire de Recherches sur la Traite
ENSAR, 65, rue de St Brieuc - 35042 Rennes Cédex
**CEA - Service de Pharmacologie et Immunologie
CEA/CEN SACLAY F 91191 Gif-sur-Yvette Cédex

ABSTRACT

The pure tetrameric form of Acetylcholinesterase (EC - 3.1.1.7) from the electric eel electrophorus electricus has been covalently coupled to oxytocin. This conjugate has been used as tracer in a heterologous competitive immunoassay. Microtiter plates coated with a mouse monoclonal anti-rabbit immunoglobulin antibody were used to separate bound and free moieties of the tracer. Acetylcholinesterase activity bound to the solid phase was measured by a colorimetric assay. The minimum detectable concentration was 0.075 pg/well (ie 1.5 pg/ml) and precision was less than 8 % at concentration above 0,15 pg/well. An extraction step improved sensitivity up to 10 times with good recoveries. To assess the validity of this assay, basal levels of oxytocin were measured during the oestrous cycle of a cow.
(Key words : oxytocin - EIA - Acetylcholinesterase - Second antibody - C18 extraction)

INTRODUCTION

Oxytocin is a nine aminoacid single chain neuropeptide with a disulfide bridge, implicated in numerous physiological mecanisms such as neurotransmission in the central nervous system (learning, memory, maternal and sexual behaviour) and hormonal regulation at the peripheral level (uterine contraction, milk ejection and

luteolysis). This last point makes the oxytocin assay a very useful tool in animal production (pregnancy diagnosis, control of sexual activity), but agricultural producers require a simple, automated technique that is sensitive enough to detect very low levels of circulating oxytocin in domestic ruminants (1 to 50 pg/ml), and that is the aim of this work.

Bioassay was the first technique used to quantify oxytocin, by measuring myometrial contraction (1-2), mammary myoepithelial contraction (3-4), milk ejection (5) or intramammary rise in pressure (6). Because of the poor reproductibility, the differences in response between organs, animals and physiological status and lack of specificity, bioassays are now considered only as tools for complementary research.

High performance liquid chromatography (H.P.L.C.) allows characterization and quantification of oxytocin, but with limited sensitivity (200 pg/ml) (7).

Several radioimmunoassay systems for oxytocin have been described in the past twenty years (8-12). Depending on the properties of antiserum, the I^{125}-labelled antigen and the extraction method used (13-14), assays with sufficient sensitivity to measure basal levels of 0,25 to 5 pg/ml have been established. However, use of radioactive tracers has a number of disadvantages including regular preparation of fresh label, disposal of radioactive waste and of national agreement.

Only two non isotopic labels have been described. An isoluminol analogue has been used in a luminescent immuno assay, but specific activity of the tracer was too low for adequate sensitivity (15). An enzyme immunoassay using peroxidase (16) and second antibody precipitation provided encouraging results with a detection limit of 25 pg/ml.

In recent years, acetylcholinesterase (AChE) from "Electrophorus Electricus" has been used as a label (17). We have shown that AChE conjuguates can be used

to quantify eicosanoids (18), substance P (19), thyroid releasing hormone (20), cyclic nucleotides (21) and opioid peptides (22), with sensitivities equal to or greater than those of conventionnal RIAs.

We report here the development of a sensitive enzyme immunoassay (EIA) for oxytocin using AChE conjugate and second antibody coated microtiter plates as solid phase, together with prior extraction to improve sensitivity. The cross reactivity of the antiserum used has been investigated in order to assess the contribution of oxytocin fragments in plasma samples and the assay has been applied to physiological samples.

MATERIALS AND METHODS

Unless otherwise stated, all reagents were from Sigma Chemical Company (P.O. Box 14508. St Louis M.O. 63178 USA).

Immunization

Oxytocin antisera were obtained by immunizing white New Zealand adult male rabbits with nine different immunogens, prepared by conjugating oxytocin (Syntocinon (200 UI/ml) Sandoz company CH 4002 Basel, Switzerland) with poly-L-arginine (Poly A), porcine thyroglobulin (Thyro) or keyhole limpet hemocyanin (KLH). For each carrier protein, conjugation was achieved using glutaraldehyde (Gluta), 1.3 dimethyl-Aminopropyl-3-ethyl carbodiimide (E.D.C.) or toluene 2.4 Di-isocyanate (T.D.I.C.) (Serva Feinbiochemica Gmbh. P.O. Box 105260 Carl-Bery-Straße 7 D-69000 Heidelberg 1) according to the methods of Dubois (23) and Geenen et al (24). Immunizations occured 4 times at weekly intervals followed by booster injections one month later and then each month. Mouse monoclonal antibody (SAL 173) against rabbit IgG was prepared in our laboratory as previously described (25).

- Enzyme Label

Acetylcholinesterase (AChE) (EC 3.1.1.7) was extracted from the electric eel *"Electrophorus electricus"*, and purified by affinity chromatography as described by Massoulié and Bon (26). The tetrameric form of AChE (G4-AChE) was prepared from the purified AChE preparation by treatment with trypsin (1μg/ml) for 18 h at 25°C in 0.1 M phosphate buffer pH 7 (1/2000 trypsin-AChE ratio (w/w)). This mixture, which contained mainly the tetrameric form, was used without further purification. Acetylcholinesterase activity was measured by the method of Ellman et al. (27). Enzymatic activity was expressed in terms of the Ellman unit (EU) as previously defined (21).

- Preparation of labelled Tracer (OXT-AChE)

Oxytocin was covalently coupled to the tetrameric form of AChE by reaction with N-succinimidyl-4-(maleido-methyl)-cyclohexane-1-carboxylate (SMCC) as previously described (19, 28). This method involved the reaction of thiol groups introduced into oxytocin using N-succinimidyl-S-acetyl-thioacetate (SATA) with a maleimide group incorporated into AChE after reaction with SMCC. To 2 mg of oxytocin (about 2 μmoles) dissolved in 0.1 M borate buffer pH 9, were added 4.26 mg of SATA (20 μmoles). After 30 min reaction at room temperature thioester was hydrolysed in presence of hydroxylamine (2,5 x 10^{-1} M) and the thiolated peptide was purified on a SEPPAK cartridge as previously described (19, 28). The thiol content of the peptide was measured using Ellman method's (27). Conjugation of thiolated oxytocin with AChE was obtained by mixing SMCC treated AChE (0,5 μmoles) with an excess of SH-oxytocin (5 nmoles) (SH-oxytocin/G4-AChE = 10). After 3 hours reaction at 30°C the conjugate was purified on a Biogel A 15 m column as previously described (19).

- Apparatus

The assay was performed using Dynatech microtitration equipment (Dynatech laboratories, Daux Road, Billingshurst, West Sussex RH 14 95 J U.K.) including an automatic plate washer (Ultrawash II) an automatic plate dispenser (multidrop) and an automatic plate reader (MR 5000) with a reading time of 1,2 s for one plate, one wavelength.

Microtiter plates (96 F immunoplate I with certificate) were from Nunc (Denmark). Enzyme activity measurements were made with a Statar III spectrophotometer (Gilford, Oberlin OH). Molecular sieve chromatography was performed with the FPLC system of Pharmacia (Uppsala, Sweden) using a Superose 6 column HR 10/30.

- Competitive Immunoassay Procedure

All assays were performed in 0,1 M phosphate buffer, pH 7,4, containing 0,15 M NaCl, 0,1 % bovine serum albumine and 0,01 % sodium azide (EIA buffer).

EIA was performed as described elsewhere (18-22). The 96-well microtiter plates were coated with mouse monoclonal antibodies specific for rabbit IgG, as previously described (25). Before use, plates were washed with 0.01 M phosphate buffer, pH 7,4, containing 0,05 % tween 20 (washing buffer, 300 µl/well, five wash cycles). Reagents were dispensed as follows : 50 µl of anti-oxytocin antiserum diluted in EIA buffer; 50 µl of standard or plasma sample and 50 µl of OXT-AChE conjugate diluted in EIA buffer (3.0 EU/ml) with a preincubation time of 0 to 48 h. The plates were covered with a plastic adhesive sheet (from Dynatech Laboratories) and incubated for 18 to 68 h at 4°C. The plates were washed and shaken to eliminate remaining buffer and 200 µl of Ellman's reagent were automatically dispensed into each well. During the enzymatic reaction the plates

were gently agitated in the absence of direct illumination. When the absorbance of the Bo wells at 414 nm reached 0.2 Absorbance Units (A.U.) (45-75 min) the absorbance was automatically measured in all 96 wells.

Calibration curves were prepared using a Log Logit transformation with the Immunofit EIA/RIA software (Beckman CA 92634 - 3100 - USA).

All measurements were made in duplicate except for Bo values which were in quadruplicate. Non specific binding was determined using an incubation mixture in which the specific antibody was replaced by EIA buffer. The minimum detectable concentration was defined as the concentration at B0 minus 3 standard deviations.

- Specificity

Compounds tested were lysine vasopressin (LVP), arginine vasopressin (AVP), isotocin, tocinoic acid, Tyr-Pro-Leu-GlyNH2, Thr4 Gly7 Oxytocin (Peninsula Laboratories Europe Ltd, Box 62, St Hellens, Merseyside UK, WA 9 3 AJ), ovine prolactine (oPRL), porcine FSH (pFSH), ovine LH (oLH), PGF2α tromethamine salt (Upjohn Ltd, Crawley, Sussex, UK), MSH and neurophysin (purified by C. Creminon, SPI, CEA/CEN Saclay F 91191 Gif sur Yvette Cédex). Cross reactivity was expressed as dose of oxytocin / dose of analog at binding of 0.5 B0 . All the results were obtained with the optimized protocol (ie delayed addition of tracer).

- Oxytocin-Free Plasma Preparation

Oxytocin-free plasma was obtained from two pools of plasma made from 15 lactating ewes and 4 lactating cows respectively. Plasma was treated with 200 mg of Norit A charcoal and mixed at 4°C for 4 hours (9). After centrifugation (3000 g, 15 min), supernatants were decanted and filtered through 20 µm disposable syringe plastic filters (Millex, Millipore corporation, Bedford Massachusetts 01730 USA).

- Recovery Studies

Oxytocin-free plasma was assayed after addition of 55, 110, 274, 548, 1095, 2190 and 5475 pg/ml of oxytocin, after dilution of plasma samples 2, 4 and 10 times with EIA buffer and after concentration 2, 4 and 10 times after extraction.

- Preparation of Biological Samples - Extraction Procedure

Blood samples were taken from the subcutaneous abdominal mammary vein of cows or the jugular vein of ewes using chilled heparinized Vacutainers (Beckton Dickinson France, Cédex 227. 38 - Grenoble-Gare). In order to limit oxytocin degradation, tubes were stored in iced water (0-4°C) before centrifugation at 4°C (3000 g during 15 min). Plasma was collected in chilled polypropylene tubes or siliconed glass tubes and stored at -20°C. Because degradation of oxytocin at -20°C is about 42 % by one year for standard (29), samples were assayed within two months. Thawed samples were recentrifugated (4°C, 3000 g, 15 min) before extraction (1 ml of plasma is needed).

Extractions were made using SEPPAK C18 cartridges (Waters. Associ. Inc. Milford, USA) according to the technique described by Schams (11) with minor modification: after acidic elution, the cartridge was rinsed one more time with 10 ml ultrapure water to eliminate acid residues that could inhibit AChE. Recoveries were 90 ± 5 % (n = 30) with oxytocin concentrations of 1 ng/ml. Under these conditions, it was possible to use the cartridge 5 times after regeneration with 2 ml methanol followed by 5 ml water (intracolumn coefficient of variation = 6,8 %, n = 30). Eluated oxytocin was dessicated in a vacuum centrifugal evaporator (Speed-Vac Savant instruments 110-113 Bi-country-blvd, Farmingdale, NY 11735, USA) and stored at - 20° C. Samples were reconstituted in EIA buffer allowing concentration up to 10 times. Blanks were prepared in order to evaluate interference due to the extraction procedure.

- Physiological Studies

Plasma samples were taken in early pregnant cows during 32 days before and after PGF2α challenges (Dinolytic - Upjohn, 256 µg i.v. bolus), which induce luteal oxytocin release without leading to luteolysis (30).

RESULTS

- Antisera

Antibodies were raised in rabbit using 9 differents immunogens obtained by different combinations between carrier proteins (KLH, Poly A, Thyro) and coupling procedures (see methods). Titres achieved by the 9 conjuguates are shown in table 1.

Because antiserum R8 (KLH - Gluta) appeared the most sensitive (B/Bo 50 % = 1,9 pg/well), it was selected to optimize the assay.

- Specificity Of The Antiserum R8 :

Cross reactivities observed with oxytocin derived peptides and other hormones using a 1/40000 dilution of antiserum R8 and the optimized assay protocol, including delayed addition of tracer, are shown in table 2. Cross reactivities never exceed 0.4 %, except 12.5 % obtained with the tripeptide tail of oxytocin (Tyr-Pro-Leu-Gly-NH_2).

- Optimization Of The Assay :

Binding of OXT-AChE to antioxytocin at 4°C comes to 95 % of equilibrium after 192 h incubation (results not shown).

Maximal enzymatic activity bound to the well never exceeded 6 % of the total activity introduced in the assay even with an antibody dilution of 1/1000.

With 1/60000 final dilution of antiserum R8, 48 h preincubation of antibody with antigen followed by 72 h incubation with tracer, standard curves with B/Bo

TABLE 1

A : Titre (x 10^{-4})

	EDC	GLUTA	TDIC
KLH	3 - [6]	4 - [5]	4 - [5]
POLY.A	0	1 - 4	2 - [6]
THYRO	3 - [9]	2 - 4	2

B : Sensitivity B/BO 50 % (pg/ml)

	EDC	GLUTA	TDIC
KLH	> 20	[2] - 6	[5] - 20
POLY.A	-	> 20	[5] - 20
THYRO	[3] - 15	7 - 20	18 - 20

- Titre is defined as the dilution that bind 50 % of the maximum enzyme activity that can be bound on the well.
- Sensitivity is defined as the amount of oxytocin required to inhibit binding of the enzymatic tracer (OXT-AChE) by 50 %.
- EDC/GLUTA/TDIC are the coupling agents used for immunogen synthesis (1.3 dimethyl-Aminopropyl-3-Ethyl carbodiimide/Glutaraldehyde/Toluene 2,4 Diisocyanate respectively).
- KLH/THYRO/POLY. A are the carriers used for immunogen synthesis (Keyhole limpet hemocyanine/Porcine thyroglobulin/Poly-Arginine respectively).
- Numbers show the minimum and maximum titre and sensitivity obtained.
- Numbers between brackets are the better results obtained.

50 % = 1,43 pg/well and detection limit of 0.075 pg/well (1,5 pg/ml without concentration) are obtained (figure 1).

Under these conditions, the intraassay Coefficient of Variation (CV) ranged from 14 % (0.075 pg/well) to 2.3 % (20 pg/well) (fig. 1). The interassay CV ranged from 7,6 % to 2,5 % (n = 10) at levels of 0.075 pg/well and 1,9 pg/well respectively.

TABLE 2

Cross reactivity of antiserum R8 with oxytocin derived peptides and other hormones

Oxytocin derived peptides and other hormones	Cross Reactivity
Oxytocin	100 %
Arginine Vasopressin	0.3 %
Lysine Vasopressin	0.15 %
Isotocin	0.15 %
Thr 4 - Gly 7 - Oxytocin	0.25 %
Tyr - Pro - Leu - Gly - NH2	12,5 %
Tocinoic Acid	$< 7.5\ 10^{-5}$ %
Neurophysin	0.4 %
Luteinizing Hormone (oLH)	0.15 %
Folliculo Stimulating Hormone (pFSH)	0.03 %
Melanocyte Stimulating Hormone (MSH)	$< 10^{-5}$ %
Prostaglandin F2α	$< 10^{-5}$ %
Prolactin (oPRL)	0.025 %

- Assay Of Plasma Samples

Direct assay of oxytocin in plasma (either cow or ewe) is not possible under these conditions because the binding of the tracer is drastically lowered in the presence of plasma.

When oxytocin is extracted from oxytocin free plasma to which standard additions have been made the resultant standard curve show no effect of our extraction procedure. Unless the supplementary washing after acidic elution is

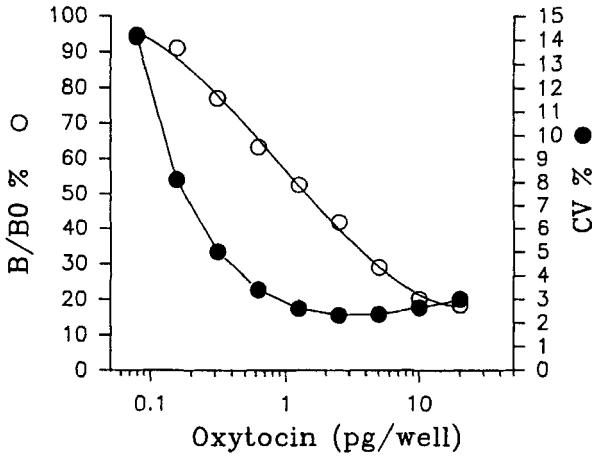

FIGURE 1 - Standard curve (○) and coefficient of variation (●) obtained with 48 h preincubation and 72 h incubation (16 replicates).

done, the pH value cannot be sufficiently buffered by EIA buffer (pH 6 instead of 7,4 needed for optimum turn over and antigen-antibody interactions). Additionnaly, it is impossible to increase the molarity of the buffer because it is detrimental to the assay sensitivity. It should be noted that the concentration of NaCl in EIA buffer is also of a great importance for a good interaction between oxytocin and antibodies and 0,15 M is needed instead of 0,4 M as described for numerous assays (18, 21) also using AChE as a label .

Recoveries after extraction range from 85 % to 118 % (fig. 2). There is good correlation between oxytocin added and recovered ($y = 1,07\ x - 13,9$; $r = 0,996$). In the same way, concentration and/or dilution of plasma samples do not significantly affect the results. There is good correlation between theoretical and assayed concentration of oxytocin ($y = 1,04\ x - 40$; $r = 0,999$) (fig. 3).

In all cases, non specific binding was less than 0,1 % of the total enzyme activity introduced in the assay.

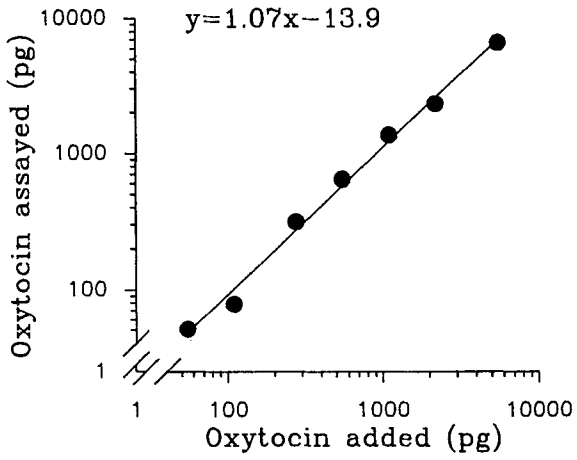

FIGURE 2 - Recovery after addition of oxytocin to a plasma sample containing 537 pg/ml of oxytocin. Oxytocin assayed is the difference between assayed concentration and initial sample concentration. The correlation coefficient between oxytocin added and recovered is 0,996.

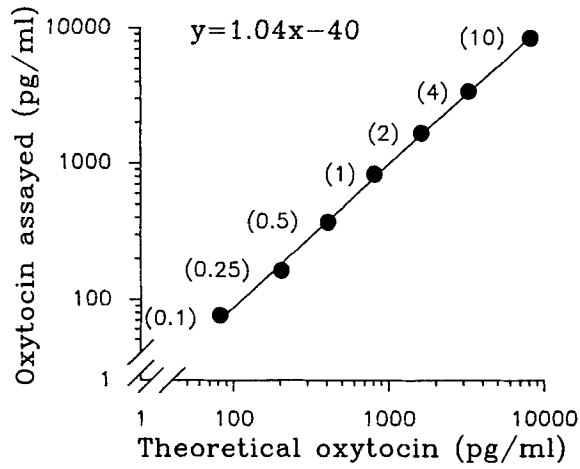

FIGURE 3 - Recovery test after reconstitution of a dryed extract of plasma sample containing 818 pg/ml with variable volumes of buffer. Numbers in parenthesis are the dilution factor with respect to original plasma. The correlation coefficient between theoretical and assayed concentration is 0,999.

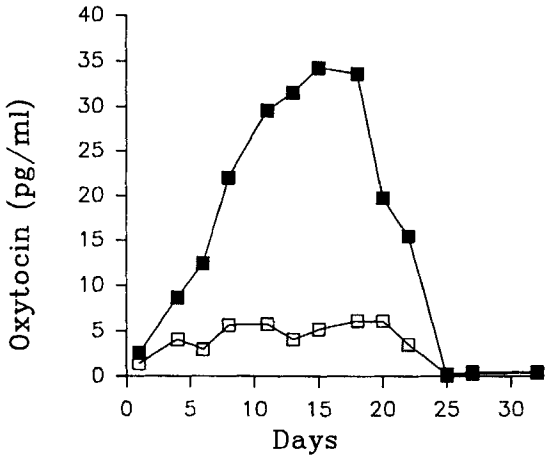

FIGURE 4 - Corpus luteum release of oxytocin after PGF2α challenges in early pregnant cows. (□) : control levels ; (■) : levels after PGF2α challenges.

- Physiological Study

The E.I.A. was used to measure oxytocin plasma levels in early pregnant cows when challenged with PGF2α (fig. 4). Basal levels ranged between 0,2 and 1,5 pg/ml. Control levels (without stimulation) increased until Day 8, remained stable until day 20 (5,7 to 6,1 pg/ml), and dropped to reach basal level at Day 25 of pregnancy. After PGF2α challenge, corpus luteum release of oxytocin increased from Day 1 (2,6 pg/ml) to day 15 (34 pg/ml) then decreased dramatically to reach basal levels at Day 25. No further reactivity have been observed during the late pregnancy.

DISCUSSION

In order to optimize antibody production 9 different immunogens obtained by combination of 3 carrier proteins (KLH, Poly A, Thyro) and 3 coupling procedures (Gluta, EDC, TDIC) were injected to 9 series of 2 rabbits. Excepting poly-arginine

immunogens which provided either no response or antisera with a very low sensitivity, we didn't observed very significant difference between the different preparations. Because of the inter-animal variability in immune response and the limited number of rabbits tested, we couldn't make conclusions as to the superiority of one immunogen over the others. It is worth noting that the poor results observed with poly-arginine immunogen conflicts with previous work since this carrier protein has been used successfully by other workers (8). EDC coupling produced good antisera as previously mentioned (10, 11, 16) but we obtained slightly better results using glutaraldehyde and we finally selected antiserum R8 (KLH - gluta) or this study because it provided the more sensitive standard curves.

For the antiserum R8, there is very low cross reactivity with other hormones and structurally related compounds. There is evidence of preferential recognition of the tripeptide tail of the molecule, while the ring gave virtually no cross reaction, as would be expected from coupling to the primary amino residue of cysteine. Such an antiserum should detect free and bound oxytocin (oxytocin-neurophysin and pro-oxytocin neurophysin precussor (31)) and inactivated oxytocin after the reduction of the disulfide bridge (32). That could explain the 0,4 % of cross reaction measured with our neurophysin preparation. Such reactivities could result in poor correlation with biological activity if oxytocin is not extracted before assay.

The enzyme coupling procedure retains immunoreactivity for the tracer as demonstrated by the fact the conjugate could be immobilized on a second antibody solid phase in the presence of specific anti-oxytocin antibodies. The reduction in tracer binding seen at high antibody concentration is due to the total immunoglobulin concentration exceeding the binding capacity of the second antibody solid phase (similar to an "hook" effect as observed in single step two site immunometric assays) (21).

Concerning the tracer, the 6 % binding observed at equilibrium reflects the percentage of bound enzyme in the tracer preparation. Here, about 94 % of the enzyme is hapten free. Nevertheless the presence of an excess of free enzyme does not create unacceptable nonspecific binding, which was consistently less than 0.1 % of total enzyme activity. This nonspecific binding corresponds to 1.6 % of maximal ligand binding which is still lower than NSB currently observed with RIAs.

Additionally to the well established strategies for increasing sensitivity (incubation at 4°C, lenghtened time of incubation, delayed addition of the tracer), AChE, which gives proportional and constant rate of degradation of Acetylthiocholine up to 40 h (at least) without down regulation, allows lengthened incubation time to compensate for decrease in signal with use of extreme dilution of the antiserum. This dilution of our antiserum increased the sensitivity and surprisingly decrease the intra and inter assay coefficient of variation. It is also possible to modify this incubation time in order to work in the most accurate and linear region of measurement of the spectrophotometer.

When plasma samples are assayed, an extraction step is needed. In fact, when direct coating of specific antibodies is made in the well, we saw only 10 to 17 % of displacement depending on the plasma origin (animal, species, physiological status) which correspond to a very classical matrix effect. So, the very low binding observed in the presence of ruminants plasma may be due to a recognizing of ruminant IgG by the more specific anti-rabbit IG monoclonal antibody. Trying to produce a anti-rabbit monoclonal IgG would be necessary if we want to conserve the principle of precoated well and the possibility of automation which is then facilitated.

When reverse phase chromatography is used there is no interference with tracer binding (column blank = 0) and recoveries after addition of oxytocin, dilution

or concentration of the sample are similar to those previously described for related peptides (11, 14, 33). Additionaly, because slopes of the standard dilution curve, limit of detection and sensitivities of the assay are quite similar when standard dilution are done in EIA buffer or in oxytocin-free plasma, it is possible to use EIA buffer for standard dilution in routine analysis without introducing a bias in the quantification, which is an advantage of this extraction procedure.

Our physiological study assesses our assay as an appropriate tool for oxytocin determination in ruminant plasma samples. We have detected basal levels in peripheral blood and the natural increase in oxytocin concentration due to corpus luteum secretion that has been described only by some authors (34, 35), as well as the dramatic increase of the circulating levels due to luteal oxytocin discharge after PGF2α challenges.

Our assay appears to be a good alternative to RIA without the drawbacks associated with isotopic tracers and provides at least in part the sensitivity needed by breeders.

ACKNOWLEDGMENTS

We would like to thank Doctors B. Breton and P. Le Bail from the INRA laboratory of fish physiology (Rennes, Campus Universitaire de Beaulieu, 35042 Rennes Cédex) for their help and stimulating discussion concerning immunogen preparation and optimization of the assay ; M.C. Nevers, C. Creminon and all the technical staff of the service of Pharmacology and Immunology (CEA/CEN Saclay) for their help and active participation in the adaptation to E.I.A. technique ; Doctors Römer and Rissi (Pharmar. Div. Preclinical Research Sandoz Company) for their generous Gift of syntocinon ; Professor J. Labussière for his support all along this work and A. Briand for expert typing of the manuscript.

Correspondence should be sent to P.G. MARNET, INRA - Laboratoire de Recherches sur la Traite ENSAR, 65, rue de St Brieuc - 35042 Rennes Cédex .

REFERENCES

(1) Holton, P. A modification of the method of Dale and Laidlaw for standardization of the posterior pituitary extract. Brit. J. Pharmac. Chemother. 1948 ; 3 : 328-334.

(2) Bisset, G.W., Haldar, J., Lewin, J.E. Actions of oxytocin and other biologically active peptides on the rat uterus. Mem. Soc. Endocrin. 1966 ; 14 : 185-198.

(3) Van Dongen, G.G. and Hays, R.L. A sensible in vitro assay for oxytocin. Endocrinology 1966 ; 78 : 1-6.

(4) Bisset, G.W., Clark, B.J., Haldar, J., Harris, M.C., Lewis, G.P., Rocha e Silva, M. The assay of milk ejecting activity in the lactating rat. Br. J. Pharmac. Chemother. 1967 ; 31 : 537-549.

(5) Cross, B.A. and Harris, G.W. The role of neurohypophysis in the milk ejection reflex J. Endocr. 1952 ; 8, 148-161.

(6) Van Dyke, H.B., Adamsons, K.J., Engel, S.L. Aspect of the biochemistry and physiology of the neurohypophysial hormones. Recent Prog. Hormon. Res. 1955 ; 11 : 1-35.

(7) Bridges, T.E. and Marino, V. A rapid and sensitive method for the identification and quantification of sub-picomolar amounts of neurohypophysial peptides. Life Sciences 1987 ; 41 : 2815-2822.

(8) Chard, T. The radioimmunoassay of oxytocin and vasopressin, rewiew. J. Endocr. 1973 ; 58 : 143-160.

(9) Gorewitt, R.C. Method for determining oxytocin in unextracted sera ; characterization in lactating cattle. Proc. Soc. Exp. Biol. Med. 1979 ; 160 : 80-87.

(10) Robison, I.C.A.F. The development and evaluation of a sensitive and specific radioimmunoassay for oxytocin in unextracted plasma. J. Immunol. 1980 ; 1 (3) : 323-347.

(11) Schams, D. Oxytocin determination by radioimmunoassay III. Improvement of subpicogram sensitivity and application to blood levels in cyclic cattle. Acta. Endocrinol. (Copenh) 1983 ; 103 : 180-183.

(12) Wathes, D.C., Guldenaar, S.E.F., Swann, R.N., Webb, R., Porter, D.G. and Pickering, B.T. A combined radioimmunoassay and immunocytochemical study of ovarian oxytocin producing during the periovulatory period in the ewe. J. Reprod. Fert. 1986 ; 78, 167-183.

(13) Choy, V.J. and Watkins, W.B. Arginine Vasopressin and oxytocin in the porcine corpus luteum. Neuropeptides 1988 ; 11, 119-123.

(14) Khan Dawood, F.S. Method of extraction and concentration of oxytocin for radioimmunoassay. In : eds. Amico, J.A. and Robinson, A.G. Oxytocin, clinical and laboratory studies. Proc. of the Second International Conference on Oxytocin, Québec, 1984. Amsterdam, N.Y., Oxford : Excerpta Medica, 1985 : 16-24.

(15) Lequin, R.M., Moss, R.J. and Van Nislen, J.W. A luminescent label for the immunoassay of oxytocin. J. Immunol. 1986 ; 7 (3) : 169-179.

(16) Yasuda, T., Mohri, Z.I., Murakami, Y. et al. Enzyme immunoassay of oxytocin. Endocrinol. Japon 1989 ; 36 (5) : 641-646.

(17) Grassi, J., Pradelles, Ph. Composé marqué par une enzyme ; son procédé de préparation et son utilisation en enzymoimmunologie; French patent CEA-IRF 83 : 13389, 1983.

(18) Pradelles, Ph., Grassi, J., Maclouf, J. Enzyme immunoassays of eicosanoïds using acetylcholineesterase as label : an alternative to radioimmunoassay. Anal. Chem. 1985 ; 57 : 1170-1173.

(19) Renzi, D., Couraud, J., Frobert, Y. et al. Enzyme immunoassay for substance P using acethylcholinesterase as label, In : eds. Sicureti, F., Vecchiet, L., Fanciullaci, M. Trends in cluster headache. Amsterd, 1987 : Elsevier : 125-134

(20) Grouselle, D., Tixier-Vidal, A., Pradelles, Ph. Enzyme immunoassays of thyroliberin (TRH) and TRH-elongated peptides in mouse and rat hypothalamus. Neuropeptides 1990 : 17, 155-162.

(21) Pradelles, Ph. and Grassi, J., Charbardes, D., Guiso, N. Enzyme immunoassays of adenosine cyclic 3'-5'- monophosphate and guanosine cyclic 3'-5'- monophosphate using acetylcholinesterase. Anal. Chem. 1989 ; 61 : 447-453.

(22) Mor, A., Delfour, A, Sagan, S. et al. Isolation of dermenkephalin from amphibian skin, a high-affinity δ-selective opioid heptapeptide containing a D-amino acid residue. 1989 ; 255 (2), 269-274.

(23) Dubois, M.P. Mise en évidence par immunofluorescence d'un décapeptide, le LH-RF, dans l'éminence médiane Ann. Histochim 1976 ; 21 (4), 269-278.

(24) Geenen, V., Legros, J.J., Hazee-Hagelstein et al. Release of immunoreactive oxytocin and neurophysin I by cultured luteinizing bovin granulosa cells. Acta. Endocrinol. (Copenhague) 1985 ; 110, 263-270.

(25) Metreau, E., Pleau, J.M., Dardenne, M., Bach, J.F., Pradelles, P. An enzyme immunoassay for synthetic thymulin. J. Immunol. Meth. 1987 ; 102, 233-242.

(26) Massoulie, J., Bon, S. Affinity chromatography of acethylcholinesterase ; the importance of hydrophobic interactions. Eur. J. Biochem. 1976 ; 68, 531-539.

(27) Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 1961 ; 7 : 88-95.

(28) McLaughlin, L.L., Wei, Y., Stockmann, P.T. et al. Development, validation and application of an enzyme immunoassay of atriopeptin. Biochem. Biophys. Res. Comm. 144 : 469-476.

(29) Robinson, I.C.A.F. Radioimmunoassay of oxytocin : the standard. In : eds. Amico, J.A. and Robinson, A.G. Oxytocin, clinical and laboratory studies. Proc. of the Second International Conference on Oxytocin, Québec, 1984. Amsterdam, N.Y., Oxford : Excerpta Medica, 1985 : 24-31.

(30) Labussière, J., Philibert, C., Combaud, J.F., Dotchewsky, D. Etude de l'efficacité de PGF2α sur l'éjection du lait au cours du cycle sexuel de la vache. Reprod. Nutr. Dev. 1982 ; 22 : 49-63.

(31) Breslow, E. Chemistry and biology of the neurophysins. Ann. Rev. Biochem. 1979 ; 48 : 251-274.

(32) Leake, R.D., Fisher, D.A. Special consideration in radioimmunoassay measurement of oxytocin in pregnancy plasma. In : eds. Amico, J.A. and Robinson, A.G. Oxytocin, clinical and laboratory studies. Proc. of the Second International Conference on Oxytocin, Québec, 1984. Amsterdam, N.Y., Oxford : Excerpta Medica, 1985 : 39-43.

(33) La Rochelle, F.T., North, W.G., Stern, P. A new extraction of Arginine vasopressin from blood : The use of octadecasilyl-silica. Pflügers Arch. 1980 ; 387, 79-81.

(34) Flint, A.P.F. and Sheldrick, E.L. Ovarian oxytocin. In : eds. Amico, J.A. and Robinson, A.G. Oxytocin, clinical and laboratory studies. Proc. of the Second International Conference on Oxytocin, Québec, 1984. Amsterdam, N.Y., Oxford : Excerpta Medica, 1985 : 335-350.

(35) Schams, D., Schallenberger, E., Meyer, H.H.D. et al. Ovarian oxytocin during the oestrous cycle in cattle. In : eds. Amico, J.A. and Robinson, A.G. Oxytocin, clinical and laboratory studies. Proc. of the Second International Conference on Oxytocin, Québec, 1984. Amsterdam, N.Y., Oxford : Excerpta Medica, 1985 : 313-334.