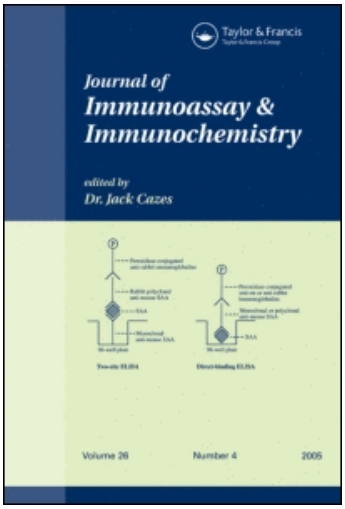

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A Luminescent Label for the Immunoassay of Oxytocin

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A LUMINESCENT LABEL FOR THE IMMUNOASSAY OF OXYTOCIN

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ABSTRACT

Chemiluminescent labels have been shown to be interesting alternatives to radioisotope labels. Disadvantages of the latter are preparation of e.g.labelled protein/peptides every four to six weeks, and problems with storage and disposal.
Amino-Butyl-Ethyl-Isoluminol(ABEI) was attached to the alpha-amino function of the N-terminal amino acid residue of oxytocin; this complex was used in immunoassays for oxytocin. This non-isotopic label did not require heating at 60°C for optimal light-signal development, a procedure usually required for chemiluminescent labels.
Standard curves were set up employing the ABEI-label on the one hand and ^{125}I-label on the other. Under identical conditions of final antibody concentration and amount of label, a comparison was made between the performance of the luminescent immunoassay (LIA) and that of the radioimmunoassay (RIA).
We conclude that the LIA systems resulted in standard curves of high precision; in comparison with RIA, the sensitivity of the LIA curves is not yet sufficient for the determination of oxytocin concentrations in e.g.human biological fuids.
Further improvements in sensitivity of the LIA systems are to be expected by selection of other luminescent labels or by the use of a more sensitive measuring device.

Keywords: chemiluminescence, oxytocin, immunoassay

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INTRODUCTION

Radioimmunoassay systems for oxytocin (OT) have been described by several groups in the past fifteen years (1-9). Depending upon the properties of the antibodies raised against the nonapeptide and the quality of the ^{125}I-labelled antigen, high quality assays have been obtained; sensitivities, defined as the minimal detectable dose, of these assays varied from 0.5 to 10 picogram peptide per tube.

The use of radioactive labels has a number of disadvantages e.g.regular preparation of fresh label, creation of radioactive waste, etc. So that there is need for reliable non-isotopic instead.

Chemiluminescent labels such as isoluminol derivates have been described for a variety of hormones and compounds but as yet not for small peptides (10-12).

We have selected the isoluminol analogue 6-(N-(4-aminobutyl)-N-ethyl)amino-2,3-dihydrophthalazine-1,4-dione, ABEI, and covalently coupled it, using a bifunctional reagent, to the N-terminal end of oxytocin. This ABEI-spacer-OT was tested with a number of available antisera.

We report a comparison of the chemiluminescent immunoassays systems with the well-established RIA-OT-system.

MATERIAL AND METHODS

Antigens:

Synthetic oxytocin (*1-9*) for labelling and standard were kindly donated by the Scientific Development Group, Organon Int.B.V., Oss, The Netherlands. (Batch STMK 12A).

For purposes of cross-reaction testing, the following synthetic peptides were also made available: OT(*1-8*), OT(*1-7*), OT(*4-8*), OT(*4-9*), OT(*6-9*), arginine vasopressine (AVP), N-acetyloxytocin, vasotocin, oxypressin and ACTH. It should be noted that the OT fragments *4-8*, *4-9*, *6-9* at the 6 position have a cystine residue (asymmetrical disulfide (13)) and the fragments *4-8* and *4-9* a pyroglutamic acid residue at the N-terminal end.

All peptides were readily dissolved in 0.25% (v/v) aqueous acetic acid to a concentration of 1 mg/mL, and an appropriate dilution series from 100 ng to 1 pg/50 uL was made from this stock solution with assay buffer: 0.1 M phosphate buffer saline (PBS) pH 7.4 containing 0.1% w/v gelatin (Sigma) and 0.1% (w/v) sodium azide (Merck).

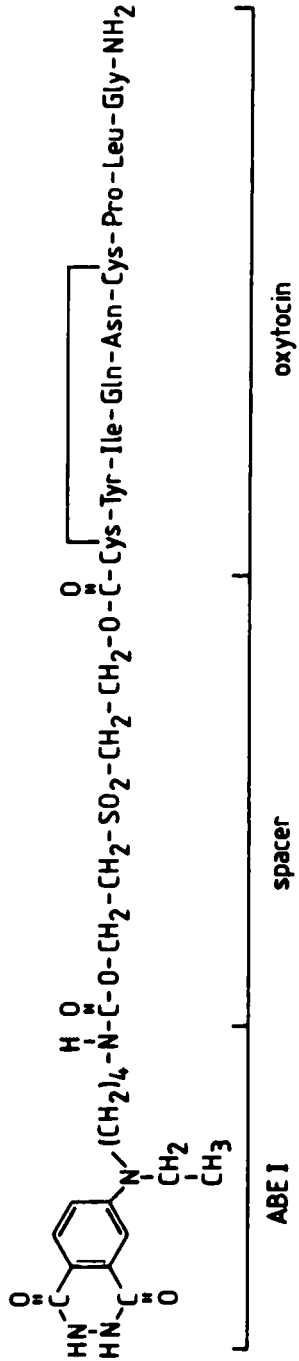

FIGURE 1: Structure of ABEI-spacer-oxytocin label.

Preparation of Isoluminol-derivate of OT:

The amino group of ABEI was linked to the N-terminal cysteine residue of OT via the bifunctional amino reactive reagent bis-(2-(succinimidooxycarbonyloxy)ethyl sulfone (14). For that purpose, 15.1 mg (0.055 mmol) of ABEI (Sigma) was reacted with 21.8 mg (0.050 mmol) of the symmetrical active ester (gift from Prof.Tesser) in 1 ml of N,N-dimethylformamide at room temperature for 1 h. Then OT was added (50.0 mg. 0.050 mmol) and the reaction mixture was stirred at room temperature for 1 h. (the pH was approx.6.8-7.0) and kept overnight in the refrigerator. The solution was then concentrated in vacuo and ether added; the crude product was purified by chromatography on a silica gel column (Merck Fertigsäule) using the solvent system 1-butanol:acetic acid:water (4:1:1, by volume). Four fractions were obtained and examined by thin-layer chromatography, electrophoresis (cellogel plate, formamide buffer of pH 5.7; 1000 Volt, 10 mA for 30 min), amino acid analysis, UV and NMR spectroscopy. The required product (see fig.1) was obtained in a yiels of ca.16 mg.

Antisera:

Polyclonal antibodies to OT were obtained in three rabbits, O-1, O-2 and O-3. Two bleedings of each rabbit, coded G and T, were kindly made available from the Netherlands Institute for Brain Research, Amsterdam. Details of the immunization procedure and properties of the antisera have been described elswhere (15).

Assay System:

Polystyrene tubes (LP3, Luckham, U.K.) were used throughout. Final volume of the incubation mixture was 0.3 mL. This consisted of 50 uL label, 150 ul assay buffer, 50 ul standard and 50 uL antiserum dilution.
The tubes were incubated at 4°C for 3-4 days. Separation of bound and free fraction was achieved by rapid addition of 200 uL of a Dextran-coated charcoal (DCC) suspension (4.0 gram of charcoal, Merck no.2186 and 750 mg of dextran T-70, Pharmacia in 100 ml assay buffer). The tubes were centrifuged for 15 min. at 4°C at 1700 x g. 200 uL of the supernatant (bound fraction) was transferred to a new series of polystyrene tubes (2174-086, Clinicon, Denmark) to develop the luminescence signal.

Development of Luminescent Signal:

To the 200 uL aliquot (B) was added 100 uL 5 M NaOH and this mixture incubated for 0-60 min. in a waterbath at 60°C. After

allowing to cool at room temperature, 100 uL of a 20 ug/mL solution of microperoxidase (Sigma, MP11) in double distilled water was added immediately prior to measurement.
While the tube was placed in the measurement position 100 uL of a 88 mM H_2O_2 solution (Merck no.822287) was rapidly added by dispenser.
The measuring apparatus for luminescence was a Luminometer model 1250, LKB Wallac, Turku, Finland. This is a single tube measuring device; light output was integrated by a Databox LKB model 1223 and recorded on a printer. The dispenser was LKB model 1291, which delivered the 100 uL hydrogen peroxide in 3 revolutions of 33 uL each.
The light output was integrated over a period of 10 sec.and recorded on a printer as mV per integration time.

RESULTS

Preliminary Experiments:

The optimal position in the OT(*1-9*) molecule, at which the derivatisation with the isoluminol label should take place, had to be determined. The following strategy was developed: 1 characterization of the available antisera with a series of fragments of and synthetic peptides related to the intact OT molecule, and 2 selection of the least damaging position for coupling the label with respect to the epitopes found.
In experiments employing ^{125}I-OT, it was found that the antibodies of antisera O-2-G and O-2-T to a very large extent were directed to the C-terminal part of OT; a small contribution of the N-terminal part and ring structure in the intact molecule could be expected because peptide *1-8* showed a crossreaction of 0.3-1.3%, while fragment (*4-9*) gave 0.01-0.04% (Table I). Peptides like arginine vasopressin (AVP), vasotocin, oxypressin, and ACTH showed negligible crossreactivity in these systems (<0.004%).
Therefore it was concluded that the most favourable position for coupling the isoluminol derivative seemed to be position 1; also, the free amino group of the cysteine residue is readily available for such manipulation.
As a pilot experiment the crossreactivity of N-acetyl-OT was tested. It was found to be 33% of the intact molecule. Introduction of a substituent at the NH_2 group at position 1 thus affects the crossreactivity of the OT-derivative but to a much smaller extent than was the case with C-terminally shortened fragments. Thus, the luminescent label was coupled to the N-alpha-amino function of OT.

TABLE 1
Cross Reaction* of Fragments.

antiserum	1-9	1-8	1-7	4-8	4-9	6-9	AVP
0-2-G	100	0.3	<0.001	<0.001	0.04	<0.001	<0.002
0-2-T	100	1.3	<0.004	<0.004	0.01	<0.004	<0.002

*expressed as mol% with respect to full reactivity of OT (1-9), intact oxytocin, at 50% B/Bo.

Development of the Luminescent Signal:

A linear relationship was found for the free, unbound ABEI-spacer-OT; peptide amounts of 10-500 pg/tube (7-350 fmol OT/tube) represented a light output of 200-2500 mV/10 sec^{-1}. In order to keep the ratio of signal to noise optimal, a concentration of 500 pg as label was chosen. Non-specific binding of the label using DCC as separating agent was 2.0% or less.
In most assay manuals for luminescent immunoassays, preincubation is recommended of the bound or free fraction aliquot with 5 M NaOH in a waterbath of 60°C for 60 min. During this incubation, an increase of the light output of the label is usually observed. However, the present ABEI-spacer-OT label did not show an increased light output upon such incubation. Therefore in all further experiments this incubation step was omitted; it should be emphasized that the addition of 5M NaOH is essential for the optimal luminescent signal.

Immunoassay:

With the ABEI-spacer-OT label (500 pg), dilution curves of antisera O-2-G and O-2-T were set up. Antisera O-2-G and O-2-T could be used at final dilutions of 1:1200 each. As expected the binding of this label with the antibodies was much lower than with ^{125}I-OT label (tabel II).
It was decided to keep an equal antibody concentration and amount of label in LIA and RIA, in order to have a valid comparison between both methods.
Standardcurves were then set up with the two antisera at 1:1200 final dilution, and with approximately 400 fmol of each label viz.ABEI-spacer-OT and ^{125}I-OT. The results are given in Fig.2 and the assay parameters in Table II (initial binding, non-specific binding, slope and ED_{50}).
As might have been expected from the pilot studies the luminescent label has a lower affinity for the antibodies than

TABLE 2

Assay parameters* of oxytocin LIA and RIA.

antiserum O-2-G:

	Bo/T(%)	NSB(%)	EDso(pg)	slope	measuring range (pg)
LIA	29	1.5	1120	-2.08	125 - 32.000
RIA	44	4.5	50	-2.11	16 - 1.000

antiserum O-2-T:

	Bo/T(%)	NSB(%)	EDso(pg)	slope	measuring range (pg)
LIA	19	1.8	1000	-1.90	125 - 32.000
RIA	48	2.0	30	-1.69	8 - 250

*after the logit-log transformation: EDso represents the amount of standard added which results in a 50% decrease of initial binding (Bo/T). NSB= Non Specific Binding.

the radioactive label. In the RIA systems the EDso's are 10-30 fold lower than in the LIA's. The measuring range for the LIA's starts at approximately 100 pg/tube of standard OT while the RIA's -under these non-optimal conditions- start at 8-16 pg/tube. It should be noted that the precision at each point of the standardcurve of the luminescent label was superior to that of the radioactive label.

DISCUSSION

Of the two OT antisera selected for these experiments, the antibodies appeared to be directed mainly against the C-terminal glycinamide (residue 9), with a small contribution made by residue 8 and the N-terminal configuration. It seemed therefore safe to couple the luminescent label, ABEI via a spacer, to residue 1. It was observed, however, that introduction at the N-alpha-position of a small substituent like an acetyl group already decreased the affinity for the antibodies to 33% of that of the intact molecule. It was therefore not surprising to find that the much larger substituent, ABEI-spacer, also reduced the affinity of the OT-label for the antibodies. Manipulation at position 1 appears to be permitted with some loss of reactivity for the antibodies used.

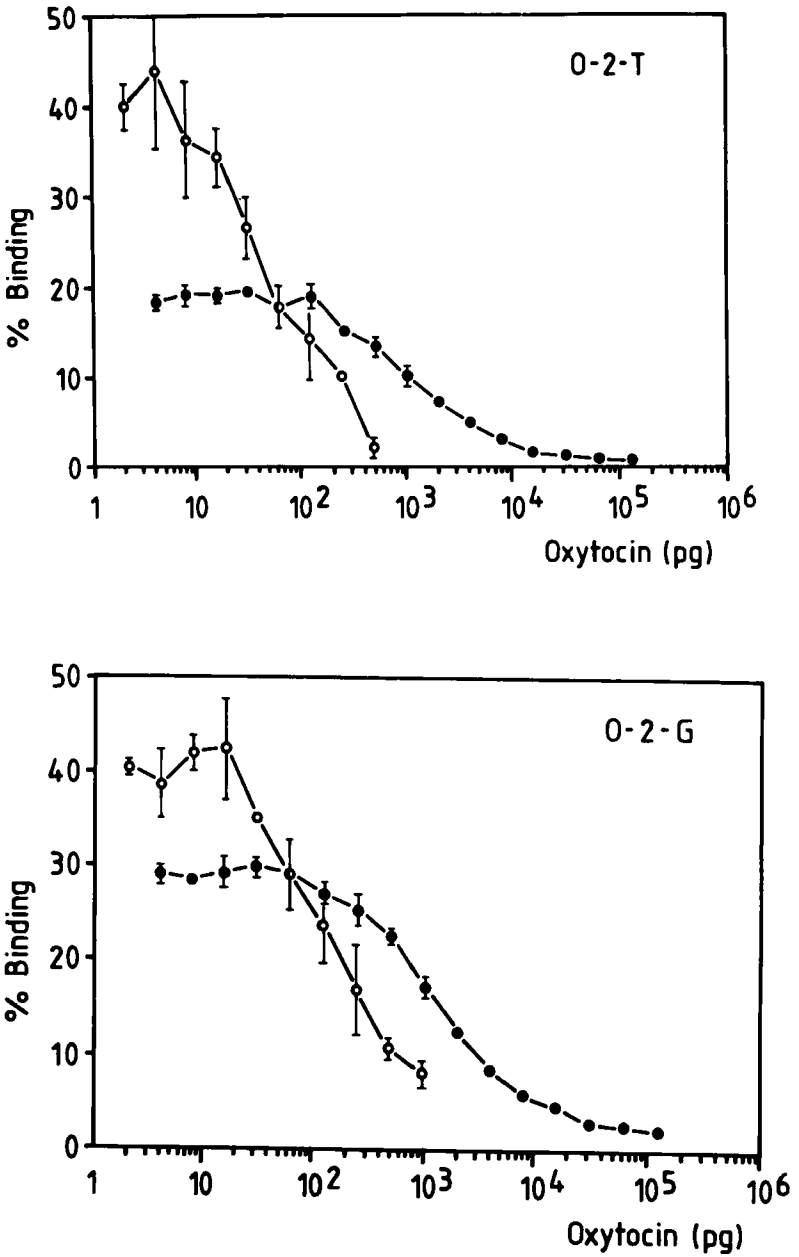

FIGURE 2: Standardcurves of antisera O-2-G and O-2-T with non-isotopic label (ABEI-OT (●) and isotopic label (^{125}-OT (○). Label concentration 400 fmol per tube; antiserum dilution 1:1200 final.

Many ABEI-labels have been prepared for compounds such as steroids, steroid-glucuronides and proteins (10-12). Most of these labels require an extensive incubation -usually 60 min.or more- at 60°C in the presence of 5M NaOH, for optimal luminescent signal development. Our label appeared not to require such a lengthy incubation as it was found that upon incubation at 60°C no further increase in signal (light output) occurred. In the presence of 5M NaOH at room temperature, the bonds of ABEI-spacer and spacer-OT most likely readily split, thereby setting free the ABEI component of the label.
Valid standardcurves could be readily obtained using ABEI-spacer-OT as a chemiluminescent label.
Under the described conditions, the RIA's still compare favourably with the LIA's, as far as sensitivity is concerned. A better precision for the LIA's was observed.
With the luminometer used as described above, we had to select relatively large quantities of luminescent label in order to have a good working range i.e.optimal signal to noise ratio. Other luminometers with greater sensitivity to measuring the light output, could already improve the LIA system because it is then expected that less label is needed for optimal binding to the antisera at dilutions greater than the ones used here.
Further improvement of the LIA may be expected from utilizing other luminescent labels like acridinium labels. Further work in these directions is in progress.

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