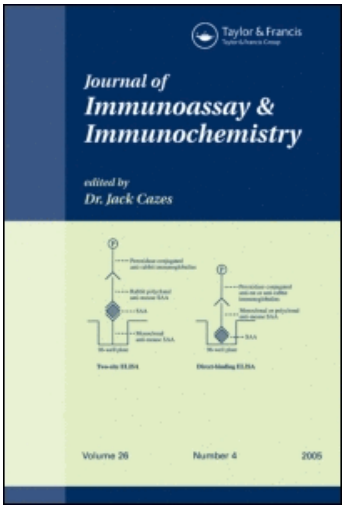

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VALIDATION IN RAT PLASMA OF A DIRECT RADIOIMMUNOASSAY FOR A LUTEINIZING HORMONE-RELEASING HORMONE ANTAGONIST (BIM 21009)

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ABSTRACT

Rabbits were immunized with [Ac-D-β-Nal[1]-, D-p-Cl-Phe[2], D-Phe[3], D-Arg[6], Phe[7], D-Ala[10]]LHRH (BIM 21009) coupled to bovine serum albumin using bis-diazotized benzidine. The best antiserum had an affinity of 5. 10^{-10} M and a specificity directed against the C-terminal part of the molecule. The antiserum was not affected by native LHRH but reacted to some extent with detergents. Assay of free-peptide plasma after gel filtration on Ultrogel AcA 34 showed apparent immunoreactivity associated with albumin and lipoproteins. The sensitivity of direct assay was 0.4 ng/ml. Measurements of BIM 21009 after s.c. injection in rats showed the resistance of the peptide to elimination. The specificity of the determinations in plasma were checked by High Performance Liquid Chromatography. (KEY WORDS : Radioimmunoassay, LHRH antagonist).

INTRODUCTION

Agonist analogs of Luteinizing Hormone-Releasing Hormone (LHRH) are commonly used in the treatment of hormonal responsive tumors such as prostatic carcinoma (1). They act by desensitizing the pituitary and inhibiting gonadotropin secretion and testi-

cular function (2). LHRH antagonists also produce chemical castration but their low potency relative to superagonists has limited their clinical application. However the development of a new generation of antagonistic LHRH analogs has provided better candidates for clinical trials (3,4). Recent studies using radioreceptor assays or high pressure liquid chromatography have demonstrated that these peptides possess longer half-lives than native LHRH or its analogs (4,5).

The LHRH antagonist [Ac-D-β-Nal^{1}, D-p-Cl-Phe^{2}, D-Phe^{3},D-Arg^{6}, Phe^{7}, D-Ala^{10}]LHRH (BIM 21009) (6) has been tested on animals in our laboratory. We have developed a radioimmunoassay suitable for assessing the peptide level in plasma in order to establish its pharmacodynamics. In this paper we report the characteristics and validation of this assay for BIM 21009 in rat plasma.

MATERIAL AND METHODS

Reagents

BIM 21009 was supplied by Laboratoires Beaufour (Dreux, France). [7-10] and [1-5]LHRH were from Bachem (Bubendorf, Switzerland) and [1-3]LHRH was from Interchim (Montluçon, France). Detergents and other peptides used in the cross-reactivity studies were purchased from Sigma (St-Louis, MO). Bovine Serum Albumin (BSA) fraction V was from Miles Scientific

(Naperville, IL) and purified BSA was from Behring (Marburg, West Germany). Freund's complete adjuvant was purchased from Difco (Detroit, MI), [^{125}I]Na was from Amersham (Cardiff, U.K.). Iodogen 1,2,4,6,tetrachloro 3α,β diphenylglycoluril, was from Pierce (Rockford, IL) and carboxymethylcellulose 52 from Whatman (Maidstone, U.K.). Aprotinin was obtained from Specia (Paris, France) and heparin from Roche (Neuilly sur Seine, France). Ultrogel AcA 34 and GF 05 were purchased from IBF Biotechnics (Villeneuve la Garenne, France). Blue Sepharose CL-6 was from Pharmacia (Uppsala, Sweden).

Peptide radiolabelling

BIM 21009 was iodinated by the iodogen method (7). Iodogen (2 µg in 20 µl dichloromethane) was placed in a polypropylene tube and the organic solvent evaporated off under nitrogen. 25 µl 0.5 M phosphate buffer pH 7.4, 5 µg BIM 21009 in 5 µl 0.1 N acetic acid and 5 µl [^{125}I]Na (18,5 mBq) were added sequentially and the mixture incubated for 10 min at room temperature. The mixture was chromatographed on a 20 x 0.7 carboxymethylcellulose (CMC 52) column and free sodium iodide was eluted with 10 mM ammonium acetate buffer pH 4.7. Iodinated peptide was eluted with 150 mM ammonium acetate buffer pH 4.7 and collected in 100 mM phosphate buffer pH 7.4 containing 1% BSA. [^{125}I]BIM 21009 (specific activity : 70 Bq/fmole) was aliquoted and stored at -80°C.

Antisera

Bis-diazotized benzidine was prepared as previously described (8). BIM 21009 was dissolved in water (2 mg/ml) and conjugated to 15 mg BSA with 10 µmoles of bis-diazotized benzidine. The pH was adjusted to 6.5 with 100 mM sodium phosphate buffer of pH 8 and the reaction mixture was incubated for 24 hours at 4°C. The BIM 21009 - BSA conjugate was purified by gel filtration on a 10 x 2.5 cm GF 05 column. The immunogen was emulsified in Freund's complete adjuvant and injected intradermally at multiple sites into adult male rabbits. Each animal was given the equivalent of 100 µg BIM 21009 in the first injection and 30 µg in booster injections. Rabbits were bled from the central ear artery and sera were stored at -20°C in 50% glycerol. Affinity constants were determined by Scatchard analysis (9).

Radioimmunoassay procedure

The diluent used in binding experiments was 100 mM phosphate buffer pH 7.4 containing 0.5% BSA, 150 mM NaCl and 0.01% NaN_3. Standard curves were established in the peptide-free plasma to compensate for the non-specific interference from plasma proteins. Standard or samples (0.1 ml) were pre-incubated with

diluted antiserum (0.1 ml) for 24 h at 4°C, then iodinated peptide (150 Bq in 0.1 ml) was added and incubation was continued further for 24 hours at 4°C. The antigen-antibody complex was separated by adding 1 ml ice-cold n-propanol and centrifuging at 2000 xg for 20 min at 4°C. Supernatants were removed and the pellets were counted in a gamma counter (Nuclear Enterprises NE 1600). Testosterone radioimmunoassays were performed with the SB-Testo-Tra kit from Oris (Gif sur Yvette, France).

Plasma fractionation

The apparent immunoreactivity of rat plasma was measured on fractions obtained by passing 10 ml plasma through a 75 x 2.5 cm Ultrogel AcA 34 column. The elution was performed at 4°C (flow rate, 17 ml/h) with 50 mM saline phosphate buffer pH 7.4. Immunoreactivity was tested on the eluted fractions. Iodinated peptide (15,000 Bq) was incubated with 10 ml of pooled rat plasma for 24 h at 24°C and chromatographed on Ultrogel AcA 34 as above. Fractions (4.5 ml) were counted for radioactivity and their absorbance at 280 nm were measured in a Beckman DU 7 spectrophotometer. Indirect estimation of the position of elution of lipoproteins was obtained by measuring the cholesterol concentration (Biotrol kit, Louvres, France) in each fractions. Albumin-depleted plasma was prepared on a 6 x 2.5 cm Sepharose Blue CL-6 column (10).

Animal studies

300 g Wistar rats were injected s.c with BIM 21009 dissolved in either saline or oil (Myglyol from Dyna, Paris, France). Blood samples were taken by retro-orbital puncture into heparinized tubes (5000 IU/ml). The samples were centrifuged for 5 min at 800 g and the plasma samples were stored at -20°C until assayed for BIM 21009 and testosterone.

High pressure liquid chromatography (HPLC)

Blood samples taken 1 hour and 10 days after peptide injection were extracted with 5 volumes of methanol-acetic acid (180 : 1). The solvent was evaporated off under nitrogen and the dried extract was dissolved in 0.1% trifluoroacetic acid (TFA). HPLC was performed using a U6K injector, two model 6000A pumps, a gradient programmer (Waters, Milford, MS) and a Gilson 201 fraction collector (Villers-le-Bel, France). A volume of 0.4 ml was injected onto a Nucleosil 5µ-C18 column and eluted at a flow rate of 1 ml/min with a 30 min linear gradient of 5-95% 0.1% TFA, 95% CH_3CN, 5% H_2O (solvent B) 0.1% TFA, 5% CH_3CN, 95% H_2O (solvent A). One ml fractions were lyophilized and dissolved in the radioimmunoassay buffer.

RESULTS

The specificity of the antibody raised using the bis-diazotized benzidine coupled immunogen is shown in Table I.

TABLE I

CROSSREACTIVITY OF BIM 21009 ANTISERUM

[Ac-D-β-Nal^1,Dp-Cl-Phe^2,D-Phe^3,D-Arg^6,Phe^7,D-Ala^{10}] LHRH (BIM 21009)	100
[Ac-p-Cl-Phe^1,D-p-Cl-Phe^2,D-Trp^3,D-Arg^6,D-Ala^{10}] LHRH	52
[Ac-D-Trp^1,D-p-Phe^2,D-Trp^3,D-Arg^6,D-Ala^{10}] LHRH	17
[D-p-Gly^1,D-p-Phe^2,D-Trp^3,D-Trp^6] LHRH	<0.001 %
[D-Lys^6] LHRH	"
LHRH	"
Thyrotropin Releasing Hormone	"
Somatostatin	"
[1-5],[1-3],[3-10] and [7-10]LHRH	"
Dodecyltrimethyl ammonium bromide	0.003 %
Triton X-100	0.002 %
Cetylpyridinium chloride	0.0003 %
Tween 20	0.0001 %

Natural peptides such as native Luteinizing Hormone-Releasing Hormone or its fragments were not recognized. Although the immunoreactivity was not changed by replacing the phenylalanine in position 7, the specificity was directed towards the C-terminal, since modifications in positions 6 and 10 were not recognized by the antibody. Some detergents were found to exhibit a relatively high level of cross-reactivity. The antiserum had an affinity of 5.10^{-10} M^{-1} and produced a sensitivity of 5 pg per assay tube and an IC_{50} (concentration producing a 50% inhibition of initial tracer binding) of 20 pg/0.1 ml.

Direct assay of peptide-free rat plasma showed a non-specific matrix effect, leading to an apparent concentration of

2.52 ± 0.45 ng/ml (x ± SEM, n=10). The nature of these non-specific effects was examined by measuring the immunoreactivity of fractions obtained after fractionation of rat plasma on Ultrogel AcA 34 column (molecular weight range : 20,000-350,000). Aliquots were assayed for peptide level, protein and cholesterol concentrations. The iodinated peptide was incubated with rat plasma and eluted under the same conditions. Figure shows that the fractions containing apparent immunoreactivity were also those which possessed high binding capacity. Interferences were found in four regions with molecular weights of 65,000, 75,000, 130,000 and ≥ 350,000. The first peak may correspond to albumin since it was eluted with the major peak of plasma protein and was absent when Sepharose Blue C-6 albumin-depleted plasma was used. The two later peaks had high cholesterol levels and were probably lipoproteins. Incubation of iodinated peptide with 10^{-4} M of unlabelled BIM 21009 did not change the extent or the nature of the binding. This protein effect was overcome and time consuming extraction of BIM 21009 from plasma avoided, by assaying the peptide from a standard curve constructed in the presence of peptide-free rat plasma. The precision profile and the standard curve of the assay are shown in Figure 2. The detection limit, defined as the dose yielding a stastically significant ($p < 0.01$) tracer displacement with a coefficient of variation <10%, was 0.40 ng/ml.

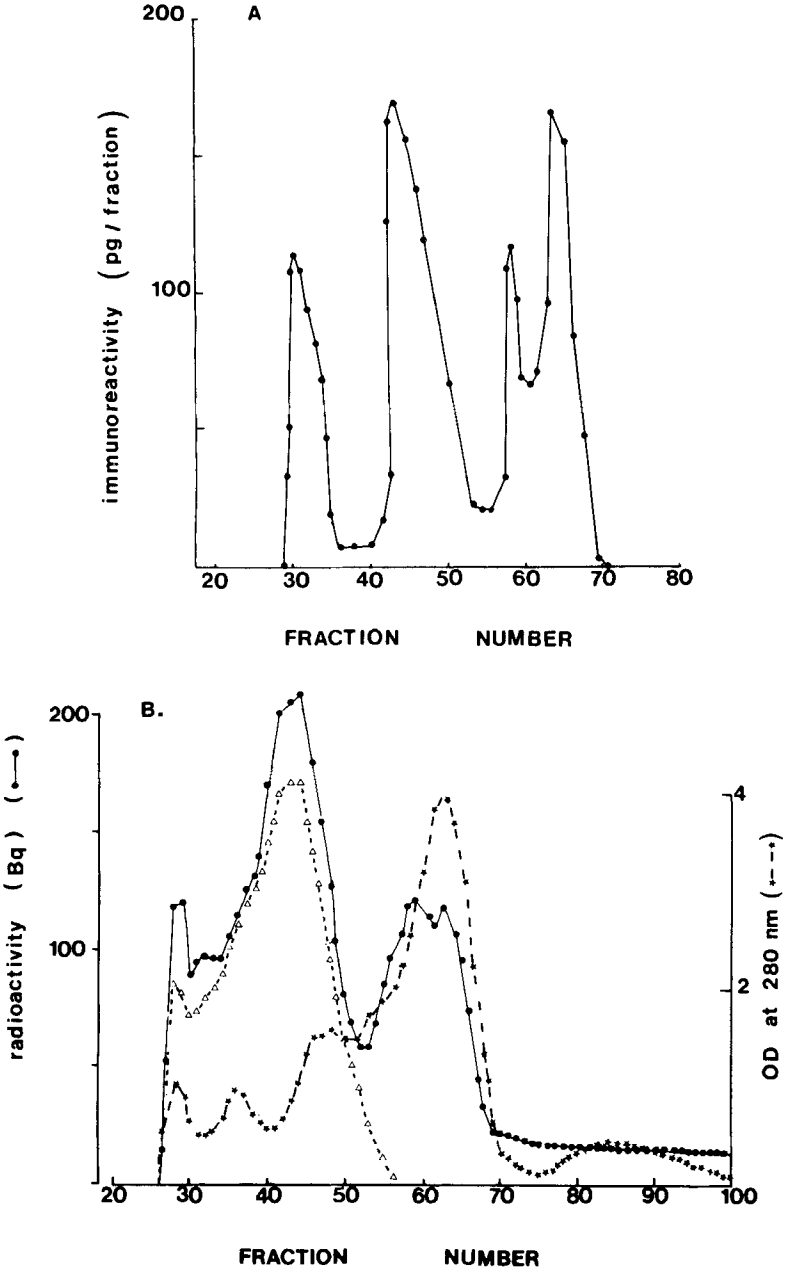

Figure 1

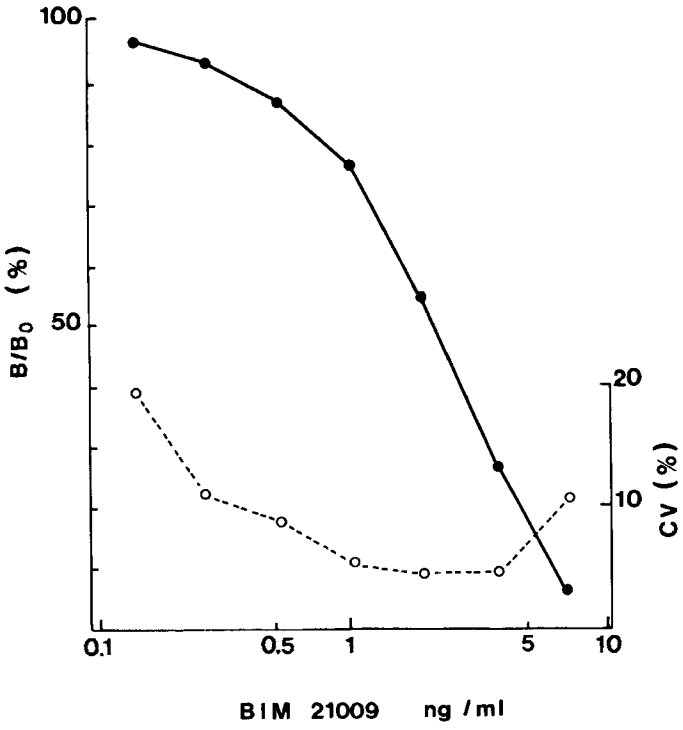

Figure 2

The intra-assay (n=10) and inter-assay (n=5) coefficients of variation were 7% and 11% at 2 ng/ml and 8% and 13% at 10 ng/ml.

Preliminary studies of peptide stability indicated that BIM 21009 incubated with rat plasma at the concentration of 8 ng/ml was not degraded after incubation for 3 hours at 4°, 20° or 37°C and after five freezing-thawing cycles, even in the absence of aprotinin.

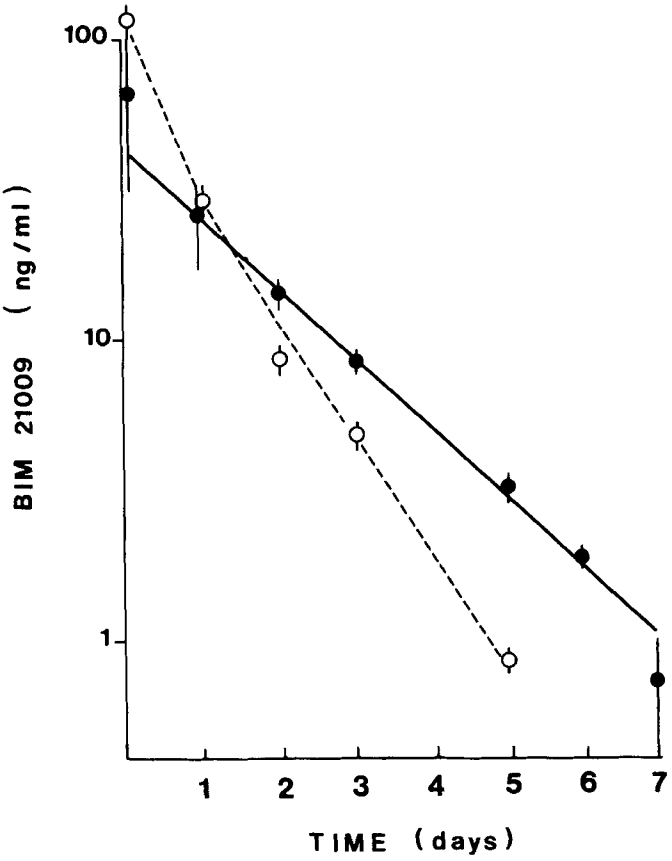

Figure 3

The plasma profile of the LHRH antagonist depended on the injection vehicle. Elimination was apparently slower when it was injected in oil (Figure 3). In both cases, the testosterone concentration was in good agreement with the peptide level and a BIM 21009 concentration greater than 5 ng/ml was required to

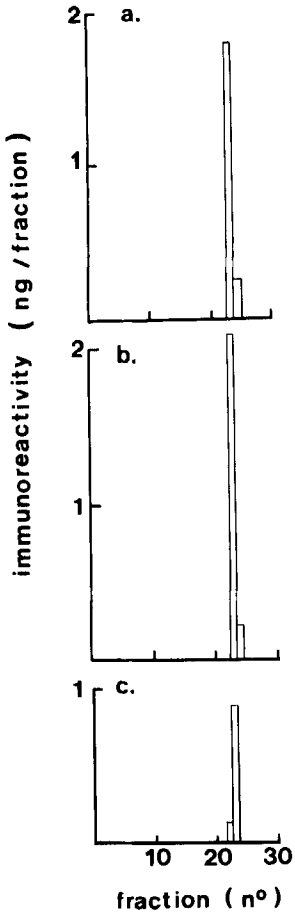

Figure 4

maintain the steroid below 0.01 ng/ml (data not shown). The specificity of the assay was demonstrated by HPLC fractionation of samples taken 1 hour and 6 days after peptide injection in oil. A single peak co-eluting with intact BIM 21009 was obtained in each case (Figure 4).

affinity binding proteins may compete with the antibody for its specific binding. It is interesting to note that, besides albumin which is known to bind LHRH or LHRH analogs (13), BIM 21009 is also extensively bound (more than 65% of the total binding) to cholesterol-associated substances, which are probably lipoproteins. As a consequence, peptide-free rat plasma must be incorporated in the standard curve. The resulting reduction in sensitivity was not a handicap, since a high dose of peptide is required to inhibit gonadal function and reduce the plasma testosterone to a castrate level.

High plasma binding, perhaps together with high tissue binding capacity, may protect against enzymatic attack. This is demonstrated by the finding that BIM 21009 has a longer half-life than do LHRH agonists (14,15). Our study confirms previous results obtained with Detirelix, a slightly different LHRH antagonist (4,5).

In conclusion, the present radioimmunoassay provides an accurate method of determining BIM 21009 in plasma and will be useful tool for establishing the pharmacokinetic profile of this new therapeutic agent.

This assay may be adapted to measurements of BIM 21009 in human plasma provided peptide-free human plasma is incorporated in the standard curve and assay specificity is verified by High Performance Liquid Chromatography.

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