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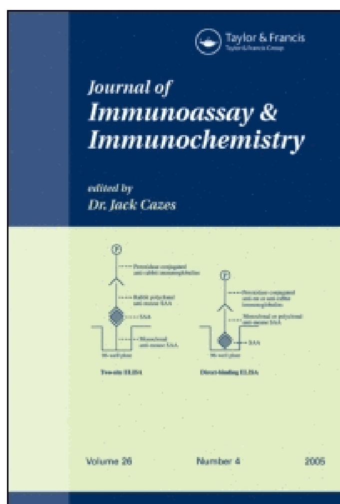

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RADIOIMMUNOASSAY OF DETIRELIX ([N-Ac-D-Nal(2)^{1},D-p-Cl-Phe^{2}, D-Trp^{3}, D-hArg(Et)$_{2}^{6}$,D-Ala^{10}]-LUTEINIZING HORMONE-RELEASING HORMONE) IN PLASMA OR SERUM.

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ABSTRACT

A procedure for the radioimmunoassay (RIA) of detirelix in plasma or serum at concentrations as low as 0.15 ng/ml is described. Antiserum was produced by deacetylation of the N-terminus amino groups of detirelix and coupling this analog to bovine serum albumin with a carbodiimide and immunizing rabbits with the resultant conjugate. For RIA, ^{125}I-labeled detirelix was used as the tracer and a double antibody procedure was used to separate the free and bound fractions. No purification of samples was required prior to RIA. Accuracy of the method was assessed by adding known quantities of detirelix to detirelix-free plasma and determining the ratio of measured to added analyte. Linear regression analysis for the concentration range 0.15-150.0 ng/ml yielded a regression equation of $y = 0.88x + 1.46$ and a correlation coefficient of 0.996. Additional validation was obtained from an in vivo study in which [^{14}C]detirelix was administered to monkeys and plasma clearance profiles were determined by RIA and an HPLC-radiochemical method. The RIA results were in good agreement with those obtained by the HPLC method (KEY WORDS: Detirelix, LHRH Antagonist, RIA).

INTRODUCTION

Detirelix (Figure 1) is a potent synthetic LHRH antagonist (1, 2, 3). It is a decapeptide in which five of the amino acids of LHRH have been replaced by D-amino acids; 3 of these are

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Figure 1:

Structure of Detirelix ([N-Ac-D-Na(2),[1] D-p-Cl-Phe[2], D-Trp[3], D-hArg(Et)$_2^6$, D-Ala[10]]LHRH).

unnatural. Detirelix is one of several antagonists currently undergoing investigation for possible application to clinical conditions involving hormone dependency (4). The procedure described here was developed to provide analytical support for various clinical, formulation and pharmacokinetic studies.

EXPERIMENTAL

Materials and Equipment

All compounds listed in Table I, with the exception of LHRH, were synthesized at Syntex. Abbreviations used in describing the structures of these analogs are given in Table 1.

The sources of the following materials are given in parentheses. Iodine-125 (NEZ-033H, New England Nuclear, Boston, MA), polyethylene-glycol 6000, bovine serum albumin and EDTA disodium salt (Sigma Chemical Co., St. Louis, MO); LHRH (Peninsula Labs, Belmont, CA); chloramine-T (Fisher Scientific Co., Fairlawn, NJ); 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce Chemicals, Rockford, IL); sodium azide (J.T. Baker Chemical Co., Phillipsburg, NJ); Sephadex QAE-A25 (Pharmacia Fine Chemicals, Piscataway, NJ); Bio-gel P-2, disposable Econo-columns, 1 x 10 cm and 1 x 50 cm (Bio-Rad, Richmond, CA). Monobasic and dibasic sodium phosphate (Mallinckrodt, St. Louis, MO); goat anti-rabbit gamma globulins (Antibodies, Inc., Davis, CA); Spectrapor 2 membrane dialysis tubing (Spectrum Medical Industries, Los Angeles, CA); and Freund's complete and incomplete adjuvant (Difco Laboratories, Detroit, MI).

The manufacturers or suppliers of the following equipment are given in parentheses. A Model DPR-6000 refrigerated centrifuge with a swing-out head (IEC/Damon, Needham, MA); a Model MM4/600 gamma counter (Micromedic Systems, Inc., Horsham, PA); a Model FC-80h fraction collector (Gibson Medical Electronics, Inc., Middleton, WI); a Model 559 spectrophotometer (Perkin-Elmer, Norwalk, CT); a Model 2600 mult-tube vortex mixer (Scientific Manufacturing Ind., Emeryville, CA).

Production of Antiserum

Both the C-terminus and N-terminus hapten-protein conjugates were synthesized by a water-soluble carbodiimide method. For the C-terminus coupling the carboxylic acid amide was replaced with a carboxyl group. The N-terminus coupling was achieved after deacetylation of the terminal amino group. The C-terminus coupling was carried out by dissolving 15.0 mg (9.0 µmole) of the carboxylic analog and 8.5 mg (45.0 µmole) of 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide•HCl in 2.0 ml of water. This

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TABLE I

CROSS-REACTIVITY DATA

Compound	Percent Cross-Reactivity
[N-Ac-D-Nal(2)^1,D-p-Cl-Phe^2,D-Trp^3,D-hArg(Et)$_2^6$,D-Ala^{10}][a]LHRH	100.00
[NH$_2$-D-Nal(2)^1,D-p-Cl-Phe^2,D-Trp^3,D-hArg(Et)$_2^6$,D-Ala^{10}]LHRH	63.0
[NAc-D-Nal(2)^1,D-p-Cl-Phe^2,D-Trp^3,D-Arg(Et)$_2^6$,D-Ala^{10}]LHRH	48.5
[NAc-D-Nal(2)^1,D-p-Cl-Phe^2,D-Trp^3,D-Lys^6,D-Ala^{10}]LHRH	4.3
[NAc-D-Nal(2)^1,D-p-Cl-Phe^2,D-Trp^3,D-hArg(Me,Bu)^6,D-Ala^{10}][b]LHRH	42.2
[NAc-D-Nal(2)^1,D-p-Cl-Phe^2,D-Trp^3,D-hArg(Et)$_2^6$,ProNEt^9][c]LHRH	1.3
[NAc-D-Nal(2)^1,D-p-Cl-Phe^2,D-PAL(3)^3,D-hArg(Et)$_2^6$,D-Ala^{10}][d]LHRH	66.1
[NAc-D-Nal(2)^1,D-p-F-Phe^2,D-Trp^3,D-hArg(Et)$_2^6$][e]LHRH	11.0
[NAc-D-p-Cl-Phe^{1,2}; D-Trp^3,D-hArg(Et)$_2^6$] LHRH	76.0
[NAc-D-Nal(2)^1,D-p-Cl-Phe^2,D-Trp^3,D-hArg(Et)$_2^6$,L-hArg(Et)$_2^8$,D-Ala^{10}]LHRH	12.7
[NAc-D-Nal(2)^1,D-p-Cl-Phe^2,D-Trp^3,D-hArg(Bu)^6,D-Ala^{10}][f]LHRH	24.7
[NAc-D-Nal(2)^1,D-p-Cl-Phe^2,D-Trp^3,D-hArg(CH$_2$)$_3^6$,D-Ala^{10}][g]LHRH	21.8
LHRH	0.037

Abbreviations - a, b, c, d, e, f, g: a, [D-Nal(2)] is 3-(2-naphthyl)-D-alanine; [D-p-Cl-Phe] is p-chloro-D-phenylalanine; [D-hArg(ET)$_2$] is N^G,$N^{G'}$-diethyl-D-homoarginine; b, [D-hArg (Me,Bu)] is N^G,$N^{G'}$-methyl,butyl-D-homoarginine; c, [ProNEt] is proline-aminoethyl; d, [D-PAL(3)] is 3-pyridyl-D-alanine; e, [D-p-F-Phe] is p-fluoro-D-phenylalanine; f, [D-hArg(Bu)] is N^G,$N^{G'}$-butyl-D-homoarginine; g, [D-hArg(CH$_2$)$_3$] is N^G,$N^{G'}$-trimethylene-D-homoarginine.

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solution was stirred for 3 hours at room temperature after which 31.0 mg of bovine serum albumin (BSA) in 1.0 ml of phosphate buffer, 0.05 M, pH 7.5 was added and stirring was continued overnight at room temperature. The N-terminus coupling reaction was carried out by dissolving 37.4 mg of BSA and 24.7 mg (15.3 μmole) of the deacetylated N-terminus analog in 4.0 ml of water. To this stirred solution was added 109.0 mg (577 μmole) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide•HCl dissolved in 1.0 ml of water. After the dropwise addition of the carbodiimide was complete (approximately 5 min.) stirring was continued overnight at room temperature. For both coupling reactions, after completion of the reaction, the entire reaction mixture was transferred to a dialysis bag and dialyzed exhaustively against saline (9.0 g/l). After dialysis was complete, the entire volume in the dialysis bag, 5.5 ml for the C-terminus conjugate and 7.5 ml for the N-terminus conjugate, was aliquoted into 0.2 and 0.5 ml aliquots, respectively, and frozen.

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Two groups of 6 New Zealand white rabbits were immunized with each of the two hapten-protein conjugates. The immunization emulsion for the N-terminus conjugate was prepared by dissolving 2.5 mg of the hapten-protein conjugate in 7.0 ml of saline (9.0 g/l) and 8.0 ml of Freund's adjuvant. For the C-terminus immunization emulsion, 1.1 mg of the protein-hapten conjugate was dissolved in 7.0 ml of saline (9.0 g/l) and 8.0 ml of Freund's adjuvant. Complete adjuvant was used for the sensitization shots and incomplete Freunds for the booster shots. Typically, 2.0 ml of this emulsion was injected into each animal at four different sites, one in each hind thigh (inside) and two at lateral sites in the intrascapular area. Each site received a cluster of five injections of 0.1 ml per injection. Booster shots were given at intervals of 4 weeks and titers were monitored at the same time. After 4-6 months, the antisera were of sufficiently good quality to be used for methods development.

Iodine-125 Labeling

The labeling reaction was carried out in the vial containing 1 mCi of iodine-125. To the vial were added, in the following order, 50 μl of phosphate buffer (0.05 M, pH 7.4), 10 μl (1 μg) of detirelix, and 20 μl (2μg) of chloramine-T. The detirelix and chloramine-T had been prepared previously in water. The reaction was allowed to proceed for 30-40 seconds and then was quenched by the addition of 500 μl of phosphate buffer (0.05 M, pH 8.0, with 1% BSA). This reaction mixture was transferred immediately to a QAE-A25 Sephadex column (1 x 8 cm) and eluted with phosphate buffer (0.05 M, pH 8.0, with 1% BSA). One ml fractions were collected in 12 x 75-mm test tubes with a fraction collector and 10 μl aliquots were used for the counting of radioactivity. A column elution profile was plotted and the fractions corresponding to the center portion of the

radioactive peak were pooled (3-4 ml). An aliquot (1.0-1.5 ml) of this pool was rechromatographed on a 1 x 30 cm Biogel P-2 column and eluted with 0.1 M acetic acid containing 0.2% BSA. Fractions (2 ml) were collected in 12 x 75 mm tubes, 10 µl aliquots were counted and an elution profile was plotted (Figure 1a). The fractions corresponding to the center portion of the major peak were pooled (10-12 ml) and aliquots of this pool were diluted in RIA buffer for use in the assay. Labeling reactions normally were carried out at intervals of 2-3 weeks.

Radioimmunoassay

Standards for the standard curve were prepared from a primary stock solution containing 0.1 mg/ml of detirelix in water. The exact concentrations of this primary standard was determined by uv absorption at 276 nm (absorptivity = 8,860). The primary standard was diluted in RIA buffer (phosphate buffer, 0.05 M, pH 7.4, with 0.5% BSA, 0.01 M EDTA, and 0.01% sodium azide) to yield a series of standards containing 10, 15, 30, 50, 70, 100, 150 pg/ 0.1 ml. These standards were stored at 4°C and were discarded after 2-3 weeks. Antiserum was diluted in RIA buffer such that 40-50% binding was achieved. This dilution ranged from 1/80,000 to 1/100,000.

The procedure for setting up the assay is as follows: add 0.1 ml of label (3000-4000 cpm), standards, unknowns, antiserum, etc., to appropriate 12 x 75 mm disposable glass tubes; add RIA buffer to adjust the final volume in each tube to 0.6 ml; cover the tubes with parafilm; vortex briefly to wash down the sides of the tubes; and then incubate the tubes overnight at room temperature.

The separation of bound from free radioactivity was carried out using a double antibody procedure. To each tube was added 0.1 ml of goat anti-rabbit gamma globulin (diluted 1:2 in RIA buffer) and 0.1 ml of normal rabbit serum (diluted 1:20 in RIA buffer). The tubes were vortexed briefly and cooled at 4°C for 4 to 5 hours. After this cold incubation the entire rack of tubes was placed in an ice bath and 0.5 ml of 10% polyethylene glycol was added to each tube. The tubes were vortexed and then placed in an ice bath for 30 min. After this, the tubes were centrifuged, the supernatant was aspirated off and the pellet was counted in a gamma counter. All counters were equipped with direct connect outputs to computers for automatic calculation of RIA results by means of a logistic program (5). Cross reactivities were determined in accordance with the method described by Abraham (6).

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Precision and Accuracy

Precision and accuracy were assessed by determining the recovery of different quantities of detirelix that had been added to plasma. In addition, a comparative study of RIA and an HPLC-radiochemical assay was carried out with specimens collected serially from cynomolgus monkeys following intravenous administration of [^{14}C]detirelix. For the HPLC radiochemical assays a C_{18} Bond Elut column was used for the preliminary purification prior to separation by HPLC (5 μ Rainin Microsorb C_{18} column; mobile phase, 40% acetonitrile in 0.05 M phosphate buffer, pH 2.6). Concentration of detirelix was obtained by dividing the radioactivity found in the appropriate HPLC fractions by the specific activity of the administered [^{14}C]detirelix.

RESULTS AND DISCUSSION

The procedure described here for the ^{125}I labeling of detirelix is a two step procedure. A rapid preliminary purification by means of the short column provides a convenient means for the elimination of unreacted ^{125}I and side products that can cause chemical degradation of the iodinated derivative. The partially purified derivative is stable for 2-3 weeks and serves as a convenient source of starting material for the second purification step, which separates nonlabeled from iodinated derivative (Figure 2b). The iodinated derivative obtained after the second purification is suitable for use in the RIA for approximately 8-10 days.

Accuracy of the overall procedure was assessed by means of experiments on the recovery of analyte that had been added to plasma (Table 2). The average intraassay coefficient of variation calculated from the results of three separate experiments (n = 2 for 10 different concentrations ranging from 0.15 to 150.0 ng/ml) varied from 0.1 to 19.9%. The interassay coefficients of variation calculated for the group mean values ranged from 3.6 to 16.6% over this concentration range. The following linear regression equation was calculated from the group means: $y = 0.88\,x + 1.46$, $r = 0.996$.

Determinations of detirelix in plasma or serum were carried out with no preliminary purification of the sample. It was necessary, however, to add an equivalent aliquot of detirelix-free plasma (either diluted or undiluted as appropriate) to all standard curve tubes in order to minimize differences in the RIA incubation media for standards and unknowns.

Antiserum was produced to both the C and N-terminus conjugates, however, the assay using the C-terminus antisera was not as sensitive (0.7-0.8 ng/ml) and the N-terminus antisera was used for development of the method described in this paper.

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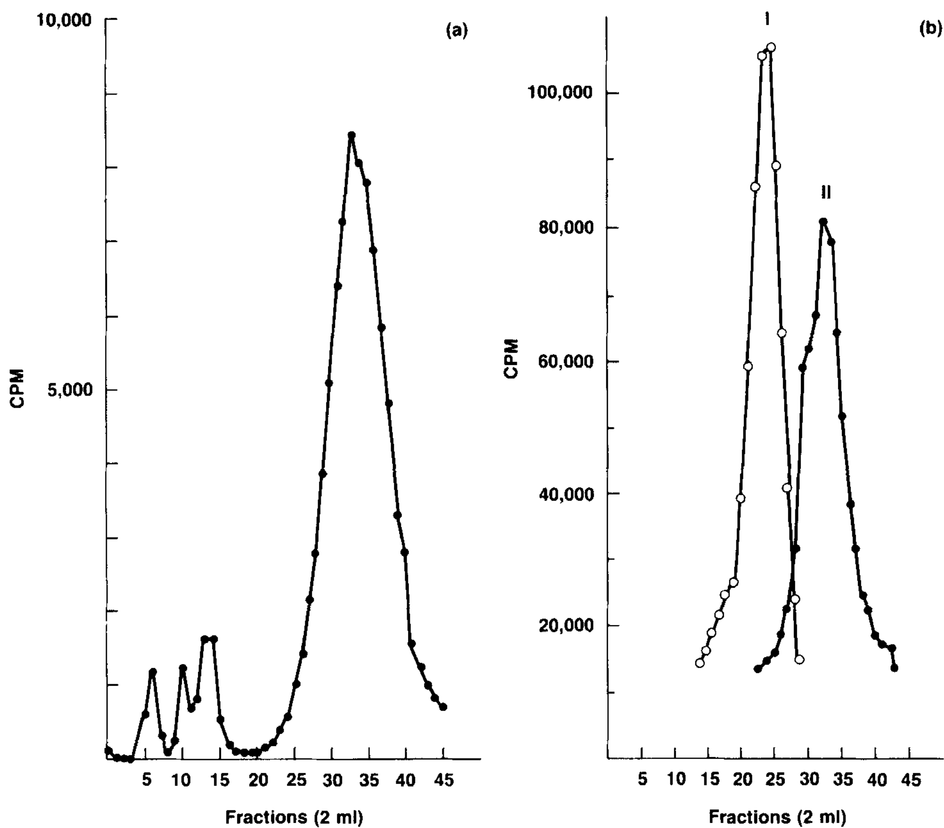

Figure 2:

Purification of iodinated detirelix. The major peak in 2a is ^{125}I-detirelix. Figure 2b shows the separation of [3H]detirelix (I) from ^{125}I-detirelix (II). Separations were carried out on a 30 cm Biogel P-2 column eluted with 0.2 M acetic acid containing 0.2% bovine serum albumin.

TABLE 2

ACCURACY AND PRECISION DATA FOR THE CONCENTRATION RANGE 0.15-150.0 NG/ML

Amount Added[1] (ng/ml)	Amount Measured (ng/ml)[2]						Group		Ratio of Measured to Added
	Expt 1		Expt 2		Expt 3				
	Mean	(% CV)[3]	Mean	(% CV)[3]	Mean	(% CV)[3]	Mean	(% CV)[4]	
0.15	0.17	(7.3)	0.13	(2.3)	0.18	(19.9)	0.16	(16.6)	1.07
0.30	0.35	(2.3)	0.29	(19.1)	0.32	(6.8)	0.32	(9.4)	1.07
0.70	0.79	(0.3)	0.67	(5.5)	0.76	(3.4)	0.74	(8.4)	1.06
1.50	1.36	(2.3)	1.58	(12.7)	1.42	(1.6)	1.45	(7.9)	0.97
3.00	3.70	(2.6)	3.20	(1.4)	3.06	(2.1)	3.32	(10.1)	1.11
7.00	7.90	(2.1)	7.60	(1.9)	7.36	(0.3)	7.62	(3.6)	1.09
15.0	13.3	(0.3)	14.3	(17.4)	14.8	(11.7)	14.1	(5.4)	0.94
30.0	28.1	(0.4)	31.1	(2.9)	30.5	(1.2)	29.9	(5.3)	0.99
70.0	71.2	(2.0)	70.2	(8.1)	78.2	(0.1)	73.2	(6.0)	1.05
150	122	(12.5)	124	(8.8)	140	(4.9)	129	(7.5)	0.86

[1] Known amounts of detirelix were added to detirlex-free plasma and assayed by this method.
[2] Values are the means of RIA duplicates, numbers in brackets are the CV's for the RIA duplicates.
[3] Intraassay.
[4] Interassay.

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TABLE 3

COMPARISON OF HPLC-RADIOCHEMICAL ASSAYS WITH RIA FOR THE DETERMINATION OF DETIRELIX IN PLASMA OF MALE CYNOMOLGUS MONKEYS GIVEN A SINGLE INTRAVENOUS DOSE OF [^{14}C]DETIRELIX

Time (hr)	Detirelix Plasma Concentrations (ng/ml)					
	Animal No. 17D		Animal No. 25B		Animal No. 657	
	HPLC-RC	RIA	HPLC-RC	RIA	HPLC-RC	RIA
0.08	746	720	1307	1000	1258	1250
0.17	415	420	729	630	836	760
0.33	279	340	572	500	521	550
0.5	223	250	507	460	410	460
1.0	131	130	306	240	211	280
2.0	29.0	66.0	125	120	99.0	130
3.0	11.5	29.0	89.0	92.0	69.0	64.0
4.0	8.7	17.0	49.0	44.0	25.0	32.0
8.0	7.7	10.4	40.0	26.0	23.0	30.0
10.0	4.8	6.0	21.0	20.0	16.0	19.6
24.0	11.5	4.6	2.0	1.6	5.0	6.3

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The data on cross-reactivity are summarized in Table 1. Of the compounds listed the deacetylated analog is a possible metabolite. ([NH_2-D-Nal(2)^1,D-p-Cl-Phe^2,D-Trp^3,D-hArg(Et)$_2^6$, D-Ala^{10}]LHRH) The extent of assay interference from this source is not known. Evaluation of metabolite interference could not be conducted because the metabolites have as yet not been identified. Independent evidence of the validity of the RIA was obtained from a comparative study in which plasma from cynomolgus monkeys that had been given [^{14}C]detirelix by intravenous injection was analyzed by RIA and an HPLC-radiochemical method. The results of this study are given in Table 3. Linear regression analysis of the data yielded the equation $y = 0.89x + 20.0$, $r = 0.988$. The good agreement between the two methods tend to indicate that there was little or no metabolite interference. The RIA method described here has been applied to pharmacokinetic, formulation and bioavailability studies, some of which have been reported elsewhere (4).

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REFERENCES

1. Vickery, B.H., McRae, G., Nestor, J.J.,, Bremner, W.J. Effects of a Highly Potent LHRH Antagonist in Rats, Dogs and Cynomolgus Monkeys. J. of Androl. 1983; 4:37.

2. Weinbauer, G.F., Surman, F.J. Aktar, F.B., Shah, G.V., Vickery, B.H., Nieschlag, E. Reversible Inhibition of Testicular Function by a Gonadotropin Hormone-Releasing Antagonist in Monkeys (Macaca fascicularis). Fert. Steril, 1984. 42 (No. 6):906-914.

3. Adams, L.A., Bremner, W.J., Nestor, J.J, Vickery, B.H., Steiner, R.A. Suppression of Plasma Gonadotropins and Testosterone in Adult Male Monkeys (Macaca fascicularis) by a Potent Inhibitory Analog of Gonadotropin-Releasing Hormone. J. Clin. Endocrinol. Metab. 1986; 62 (No. 1):58-63.

4. Pavlou, S.N., Wakefield, G.B., Island, D.P., Hoffman, P.G., LePage, M.E., Chan, R.L., Nerenberg, C.A., and Kovacs, W.J. Suppression of Pituitary-Gonadal Function by a Potent New Luteinizing Hormone-Releasing Hormone Antagonist in Normal Men. J. Clin. Endocrinol. Metab. 1987; 64:931-936.

5. Rodbard, D., Hutt, D., Faden, V.B., and Huston J.C. In RIA and Related Procedures in Medicine, International Atomic Energy Agency, Vienna, 1974; 165-192.

6. Abraham, G.J. Solid-Phase Radioimmunoassay of Estradiol-17β. J. Clin. Endocrinol. Metab. 1969; 29:866-870.

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