Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 491–496. Pergamon Press. Printed in Great Britain

A DIRECT RADIOIODINATION TECHNIQUE FOR THE RADIOIMMUNOASSAY OF 17α-ETHYNYL, 17β-HYDROXY-4-ESTREN-3-ONE*

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(*Received* 26 *February* 1975)

SUMMARY

A new technique is described for the direct labelling of norethisterone with ^{125}I for use in radioimmunoassay. This method allows the preparation of tracers possessing both high specific activity and unaltered immunological properties. The steroid is radioiodinated in the presence of H_2O_2 using acetic acid as the solvent, in a sealed vial heated at 100°C. The reaction mixture is then chromatographed on bidimensional t.l.c. and six major radioactive spots are separated; only one of these fractions possess immunoreactivity. When submitted to repeated t.l.c. it behaves as a single compound, possibly a monoiodinated derivative, substituted in Ring A.

The new tracer was compared in RIA with both the tyrosine-iodinated and the tritiated tracers. The standard inhibition curve obtained with the new labelled ligand had the same slope as that obtained using the tritiated one; on the other hand the slope of the curve obtained with the tyrosine iodinated derivative was slightly higher than that of the directly iodinated one.

The results obtained indicate that the possibility exists of radioiodinating steroids without losing their immunoreactivity.

INTRODUCTION

Radioimmunoassay techniques utilizing tracers labelled with ^{125}I for the measurement of microquantities of both natural and synthetic steroids in body fluids, offer a series of advantages over methods based on the use of tritium labelled ligands. The major advantage of radioiodine is the possibility of achieving a higher specific activity which allows both a higher sensitivity and a considerable reduction in the quantity of labelled ligand utilized. Furthermore, in spite of its shorter half life, ^{125}I provides easier, more rapid and less expensive assays [2]. Finally, this procedure permits the monitoring of procedural losses through the addition of tritium labelled, high specific activity, internal standard steroids.

The major disadvantage of this technique has been the practical impossibility of directly iodinating the steroid. Radioiodination must be carried out on either tyrosine methylester [3] or histamine [4] derivatives of the steroid. Derivatives are usually formed utilizing the same position where the steroids are conjugated with BSA for the preparation of the corresponding antisera. In this way the structure of the immunoreactive portion of the steroid molecule is not altered and antigen specificity is retained.

Theoretically steroids possessing a phenolic Ring A can easily be iodinated using simple reaction conditions. However, oestrogens iodinated in this way lack immunoreactivity, since substitution of a hydrogen with an iodine in Ring A may modify steroid configuration [5, 6]. Consequently, physico-chemical properties of such iodinated compounds will differ from those of the standard steroid.

Following failure of the attempt to obtain tracers by direct iodination of phenolic steroids, no further efforts were made to explore other possibilities to synthesize directly iodinated steroids for use in RIA. We have recently begun to reinvestigate this subject and wish to report results obtained with a direct iodination procedure applied to the synthetic steroid norethisterone, which did not result in any apparent loss of its ability to react with the corresponding antiserum when compared in a radioimmunoassay utilizing either a tritium labelled tracer or an iodinated ligand obtained via the preparation of the 3-tyrosine methyl ester derivative.

* Presented in part at the IV International Congress on Steroid Hormones, Mexico City, DF, Mexico (2–7 September 1974) [1].

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Abbreviations. The following abbreviations and trivial names have been used throughout the present paper: Ethynyl oestradiol, 17α-ethynyl-1,3,5(10)-oestratriene-3, 17β-diol; Ethyl estrenol, 17α-ethyl-4-oestren-17β-ol; Ethyl norgestrienone, 13β-ethyl, 17α-ethynyl, 17β-hydroxy-4,9(10), 11-gonatrien-3-one; Lynestrenol, 17α-ethynyl-4-oestren- 17β-ol; Norethisterone, 17α-ethynyl, 17β-hydroxy-4-oestren-3-one; Norethisterone acetate, 17α-ethynyl, 17β-acetoxy-4-oestren-3-one; Norethinodrel, 17α-ethynyl, 17β-hydroxy-5(10)-oestren-3-one; Norgestrel, 13β-ethyl, 17α-ethynyl, 17β-hydroxy-4-gonen-3-one; 19-nor-Androstenedione, 4-oestren-3,17-dione; Progesterone, 4-pregnen-3,20-dione; Testosterone, 17β-hydroxy-4-androsten-3-one. RIA, radioimmunoassay; t.l.c., thin-layer chromatography; S.A., specific activity; TME, tyrosine methyl ester; BSA, bovine serum albumin; U.V., ultra violet.

MATERIALS AND METHODS

Reagents. All organic solvents used were purchased from Merck A. G., Darmstadt, Germany and were redistilled prior to use. Steroids used as a standard were obtained through the courtesy of Dr. G. Sbarigia, Istituto Terapeutico ORMA, Pomezia, Italy. They were all recrystallized prior to use and their purity checked by measuring the melting point.

T.L.C. Silica gel plates (20 × 20 cm., thickness 0.25 mm) were purchased from Merck A. G. Darmstadt, Germany. The following systems were used at room temperature. A: benzene–acetone (92.5:7.5 v/v), B: benzene–acetone (70:30 v/v), C: benzene–acetone (80:20 v/v), D: chloroform–methanol (95:5 v/v), E: chloroform–ethyl acetate (95:5 v/v).

Direct iodination of the steroid. Radioiodination was carried out in a 1 ml vial of pyrex glass sealed after addition of all reagents in the following order: (a) Three μmol of norethisterone dissolved in 100 μl of glacial acetic acid; (b) Ten μl of [^{125}I] NaI standard solution* corresponding to 1 mCi of ^{125}I, diluted in 15 μl of glacial acetic acid; (c) One hundred μl of an H_2O_2 solution in glacial acetic acid, containing 0.1 μmol of concentrated (31.5%) H_2O_2 per ml.

The sealed vial is heated in oven at 100°C for 2 h; the reaction mixture is then chromatographed on bidimensional t.l.c. in system A for three consecutive runs on each side.

Preparation of the tyrosine methyl ester derivative of norethisterone. The 3-immino oxiacetic acid intermediate† was synthetized according to Erlanger *et al.*[7], except for minor modifications. The reaction product, with an 86% yield, was recrystallized in both benzene and ethyl acetate (melting point 158–159°C).

The carboamidic bond was obtained according to the method of Oliver *et al.*[3] using the following specifications: 1.35 mmol of tyrosine methyl ester‡ and an equal amount of triethylamine were stirred in 11 ml of CH_2Cl_2 at 10°C for 30 min; 1.35 mmol of norethisterone 3-immino oxiacetic acid were then added and the mixture brought to 10°C when 1.8 mmol of N,N'-dicycloexylcarbodiimide were added.

After 30 min the temperature of the mixture was raised to 10°C and the reaction continued for an additional 24 h. The mixture was then filtered and the supernatant evaporated under reduced pressure and chromatographed on t.l.c. in system B. Two major compounds were separated in this way, both giving a positive nitroso-naphtol reaction [8], the two fractions were co-crystallized from ethanol with a 30% yield.

Iodination of norethisterone tyrosine-3-methyl ester. Iodination of norethisterone-3-TME was carried out according to the method of Hunter and Greenwood[9], with the following specifications: 2 μg of crystalline norethisterone-3-TME were dissolved in 5 μl of ethanol and 40 μl of dioxane were added. To this mixture 1 mCi of [^{125}I] NaI was added, diluted in 25 μl of 0.05 M phosphate buffer followed by 20 μg of chloramine T dissolved in 5 μl of the same buffer. After stirring at room temperature for one min, the reaction was stopped by adding 50 μg of sodium metabisulfite in 5 μl of the phosphate buffer. Following this, 250 μg of KI in 5 μl of the same buffer were added and the mixture extracted twice with 1.5 ml of ethyl ether; the ether phase was evaporated and chromatographed on t.l.c. in system C. Four major radioactive spots were separated in this way, two of them (the more polar) possessing the same order of immunoreactivity and a S.A. of some 100 Ci/mmol. The less polar of the two products was selected for RIA because of a slightly higher binding capacity.

Preparation of anti-norethisterone serum. Norethisterone 3-immino oxiacetic-BSA derivative was prepared according to the method of Erlanger *et al.*[7]; the steroid to BSA molar ratio was 25.8, as determined by U.V. spectroscopy. Rabbits were immunized at two week intervals by multiple subcutaneous injections of 2 mg of the steroid protein conjugate emulsified in complete Freund's adjuvant.

Procedure for radioimmunoassay. "Tritiated tracer". RIA utilizing [^{3}H]norethisterone§ was carried out according to the specification of Brenner *et al.*[10]. Counting conditions were those described by Van Damme *et al.*[11]. "Directly iodinated tracer". Conditions for the assay were analogous to those employed for the tritiated tracer. Separation of bound to free ligand was obtained in this case according to the method of Desbousquois and Aurbach[12]; the residue was counted in a gamma spectrometer. "Tyrosine-iodinated tracer". With the exception of the temperature at which reaction took place (25°C), the incubation time (24 h) and the pH of the medium (pH 8), the conditions were the same as described for the other iodinated ligand.

RESULTS

When the reaction mixture obtained at the end of the iodination procedure was chromatographed on t.l.c. in system A, six major radioactive spots were developed, as indicated in Fig. 1.

Acetone eluates of individual radioactive products were further purified on t.l.c. system D and E and tested for their binding capacity against an anti-norethisterone serum. It appears from Fig. 2 that the only fraction possessing immunoreactivity was that

* [^{125}I] NaI (S.A. approximately 100 mCi/ml) was purchased from the Radiochemical Centre, Amersham (Bucks.), England.

† The amino oxiacetic acid necessary for the reaction was purchased from K & K Laboratories Inc., Plain View, N.Y., U.S.A., as its hydrochloride.

‡ L-tyrosine methyl ester hydrochloride was purchased from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.

§ Uniformly labelled [^{3}H]-norethisterone, with a S.A. of 20 Ci/mmol, was obtained through the courtesy of Biological Concepts Inc., New York, N.Y., U.S.A.

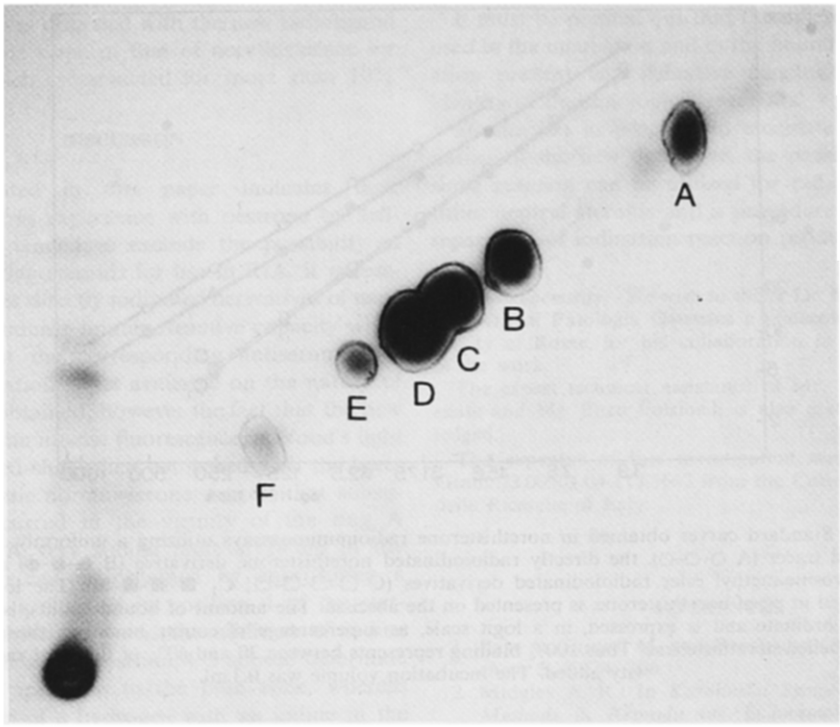

Fig. 1. Autoradiography of a bidimensional thin layer chromatogram in system A, separating labelled products formed following the new direct radioiodination procedure for norethisterone.

indicated as B. Fraction A had the same polarity of inorganic iodine and was not tested.

At a final dilution of 1:90,000 the antiserum is capable of binding 36% of the 15,000 c.p.m. of fraction B added to each tube, whereas at the dilution of 1:25,000 the binding capacity is 61%.

The binding capacity of fraction B has been tested (at 50% of binding) with a total of 8 individual anti-NET-3-BSA sera prepared in three different laboratories* at antisera dilutions varying between 1:10,000 and 1:65,000.

The nature of the immunoreactive fraction B remains unknown; it is probably composed of only one iodinated norethisterone derivative since it behaved as a single peak in t.l.c. system A and D. The compound showed an intense fluorescence in Wood's light and exhibited a maximum absorption in the U.V. spectrum at 252 nm with a red shift of 12 nm when compared with authentic norethisterone.

Stability of Compound B was tested over a period of 60 days by submitting the iodinated derivative, in both the labelled and unlabelled form, to t.l.c. in system A for three consecutive runs; both fractions behaved as a single compound. Following elution of the labelled fraction, more than 95% of the total radioactivity remained associated with the steroid.

In order to assess the suitability of the new iodinated tracer for the radioimmunoassay of norethisterone, this ligand was compared in a standard

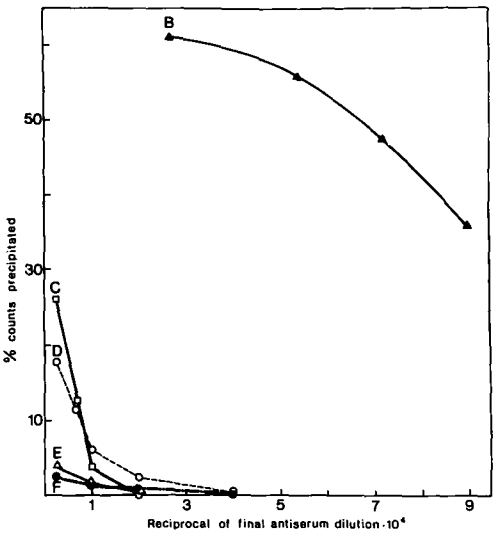

Fig. 2. Dose-response curves obtained in a norethisterone radioimunoassay utilizing as tracers the radioiodinated derivatives shown in Fig. 1. Curves were constructed by plotting dilutions of the anti-norethisterone serum against percent of labelled product bound. Counting was continued in all instances until a standard error of 1% was obtained. Compound A, exhibiting on t.l.c. the same Rf of inorganic iodine, was not assayed.

* Anti-NET-3-BSA sera were also obtained through the courtesy of Dr. F. A. Kincl, Biological Concepts Inc, N.Y., USA and Miss S. Morris, the Tenovus Institute, Cardiff, U.K.

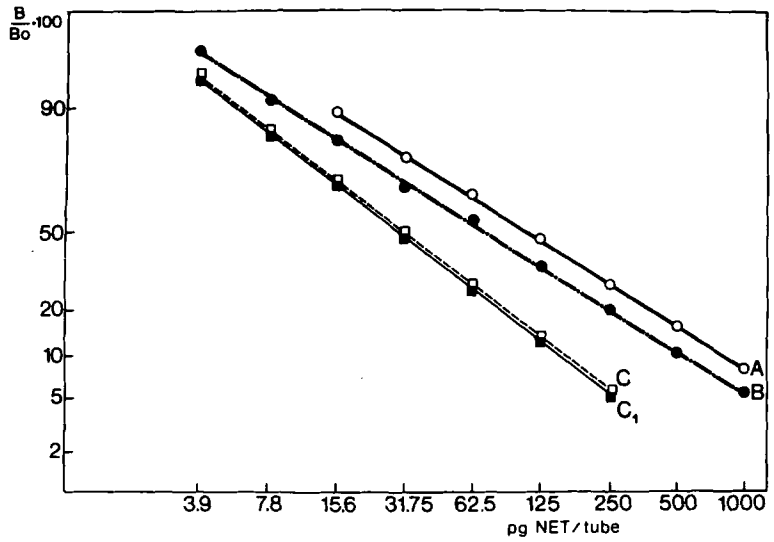

Fig. 3. Standard curves obtained in norethisterone radioimmunoassays utilizing a uniformly labelled tritiated tracer (A O–O–O), the directly radioiodinated norethisterone derivative (B ●–●–●) and the two tyrosine-methyl ester radioiodinated derivatives (C □–□–□–□; C_1 ■–■–■–■). The log dose, expressed in pg of norethisterone, is presented on the abscissa. The amount of bound counts, is shown on the ordinate and is expressed, in a logit scale, as a percentage of counts bound in the absence of unlabelled norethisterone. Thus 100% binding represents between 30 and 40% of the total radioactivity added. The incubation volume was 0.3 ml.

curve assay, with both the tyrosine iodinated and the tritiated tracers. The results of this experiment are shown in Fig. 3.

As indicated in Fig. 3, no difference was observed in the slope of the standard curves obtained with the directly iodinated compound (indicated as B) and the tritium labelled one (indicated as A). Final antiserum dilutions used were 1:30,000 for tracer A and 1:75,000 for tracer B. C and C_1 are the two tyrosine methyl ester derivatives. They are displaced by cold NET more easily than A or B.

Ten standard curves were prepared over a period of 10 months to test the reproducibility of the new radioimmunoassay. Results are given in Table 1.

Table 1 indicates that good reproducibility was obtained even at a level of less than 10 pg. The specificity of the new tracer for norethisterone RIA was tested by measuring interference with the assay of a variety of natural and synthetic steroids, utilizing for comparison the tyrosine iodinated ligand. In all instances multiple dose percent binding curves were constructed and crossreactivity was determined at 50% of binding. Results of this experiment are shown in Table 2.

Data of Table 2 clearly indicates that the new iodinated tracer possess the same order of specificity of the tyrosine iodinated derivative. The only two exceptions were norethinodrel, which crossreacts more when using the TME derivative and ethynyl oestradiol which has a higher affinity for the directly iodinated tracer.

Table 1. Assay reproducibility of norethisterone standard curve in assays performed over a period of ten months

Norethisterone added (pg)	Percent bound*	95 percent confidence limits
3.9	94.78	92.97–97.29
7.8	89.50	87.96–91.04
15.6	82.90	79.90–85.89
31.2	67.17	64.50–71.84
62.5	52.23	50.51–53.94
125	36.24	34.65–37.83
250	21.27	19.51–24.07
500	11.91	10.46–13.35
1000	6.16	4.84–7.48

* Each value represents the mean of ten determinations, carried out in a total vol. of 0.3 ml.

Table 2. Specificity of the new directly iodinated tracer in the radioimmunoassay of norethisterone compared with that obtained with the tyrosine methyl ester derivative

Steroid assayed	Percentage of crossreaction* Directly iodinated tracer	Tyrosine methyl ester derivative
Ethylnorgestrienone	<0.1	<0.1
Ethylestrenol	23.8	20.5
Ethynyl oestradiol	11.2	6.5
Lynestrenol	23.4	19.6
Norethinodrel	38.5	53.4
Norethisterone acetate	102.6	99.8
19-nor-Androstenedione	24.2	17.1
Norgestrel (racemic)	97.3	95.4
Progesterone	<0.1	<0.1
Testosterone	<0.1	<0.1

* Figures represent the mean of a minimum of three individual assays.

Standard curves obtained with the new radioligand showed the same slope of that of norethisterone for all steroids which crossreacted for more than 10%.

DISCUSSION

Data presented in this paper indicates that, although previous experience with oestrone by Jeffcoate *et al.*[6] tended to exclude the possibility of directly iodinating steroids for use in RIA, it is feasible to synthesize directly iodinated derivatives of neutral steroids retaining immunoreactive capacity when assayed against the corresponding antiserum. Not enough information is yet available on the nature of the derivative obtained; however the fact that the new product shows an intense fluorescence in Wood's light and a 12 nm red shift when compared with the spectrum of authentic norethisterone suggest that substitution has occurred in the vicinity of the ring A double bond. A very similar shift of 14 nm in the same direction can be obtained by substituting a hydrogen with a chlorine in position 4 of the steroid [13]. On the other hand the addition of iodine to the double bond in position 4–5 should determine a shift of the spectrum to the blue zone, whereas the substitution of a hydrogen with an iodine in the steroid lateral chain or its saturation, partial or total, should not influence the spectrum above 220 nm [13]. The hypothesis of a substitution in the steroid moiety rather than in the lateral chain is further substanciated by the fact that the same radioiodination procedure can be utilized for the synthesis of immunoreactive iodinated testosterone derivatives [14].

The present radioiodination technique combines the advantage of a simple labelling procedure with that of possessing cross reactivity of the same order of magnitude of that obtained with the TME derivative. The S.A. of the product obtained is sufficiently high: even assuming that all the norethisterone incubated is present in fraction B, the S.A. of the derivative is still in the order of 100 Ci/mmol.

The antiserum utilized in the present investigation binds with the same avidity the new iodinated tracer and the TME iodinated derivative. The different slope of curves C and C_1 when compared with A and B, may be due to the more complex nature of these derivatives possessing an aminoacid bound to the steroid moiety at the same position where the BSA derivative used to raise the antiserum is conjugated.

It must be pointed out that the different conditions used in the incubation and in the bound to free separation prevent any definitive conclusion about the identity of immunological reactions.

Studies are in progress to elucidate the chemical nature of the new derivative, the possiblity that the same reaction can be utilized for radioiodination of other neutral steroids and a procedure for a simpler separation of iodination reaction products.

Acknowledgements—We wish to thank Dr. Michele Ermini, Cattedra di Patologia Ostetrica e Ginecologica IIa, University of Rome, for his collaboration in certain phases of the work.

The expert technical assistance of Mr. Enzo D'Intinosante and Mr. Enzo Polsinelli is also gratefully acknowledged.

The expenses of this investigation were defrayed by Grant 73.00501.04-115.5662 from the Consiglio Nazionale delle Ricerche of Italy.

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