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THE USE OF IODINATED STEROID AS RADIOLIGAND FOR TESTOSTERONE RADIOIMMUNOASSAY

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SUMMARY

^{125}I Testosterone prepared by simple melting method [1] was purified by repeated thin layer chromatography and used as a radioligand for testosterone radioimmunoassay. The criteria of the method were evaluated and compared with those obtained using tritiated radioligand.

INTRODUCTION

^{125}I (or ^{131}I) Iodinated tyraminyl or histaminyl derivatives of various steroids have been used as alternative radioligands to tritiated compounds in steroid radioimmunoassay. The disadvantages of such iodine labelling, i.e. the requirement of derivative synthesis, its iodination followed by chromatographic removal of excessive iodide as well as short life of isotope are compensated by much easier and cheaper dosimetry and increased sensitivity. Considering the direct (i.e. in steroid nucleus) iodination, the usual objections concern the assumed instability of such compounds and the fact that presence in the molecule of bulky iodine atoms would alter possibly its immunoreactivity [2, 3].

Recently, a simple method of direct iodination based upon melting of an excess of steroid with Na^{125}I has been reported [1]. Iodinated oestradiol, testosterone and even dihydrotestosterone have been prepared in a reaction of unclear mechanism. However, only the oestradiol derivatives have been characterized more in detail.

Repeating the procedure with testosterone, we have found the technique well reproducible and the product so stable (4 weeks standing in air at room temperature did not change considerably the radioactivity distribution on chromatogram) that it seemed promising to test its use for radioimmunoassay as a radioligand.

MATERIALS AND METHODS

The radiochemicals were obtained from Radiochemical Centre, Amersham. [1,2-^{3}H]-Testosterone, specific radioactivity 2062 GBq·mmol^{-1} (56 Ci·mmol^{-1}) was purified by paper chromatography in system cyclohexane–toluene–methanol–water (v/v), its radiochemical purity exceeded 96%. Carrier free Na^{125}I was used.

Radioactivity of ^{3}H was measured on Betaszint BF 5000 (Berthold and Frieseke) liquid scintillation spectrometer, using the external standard channel ratio method for disintegration computing. The scintillation fluid consisted of 4 g PPO, 60 mg POPOP and 20 ml of methanol in 1 liter of toluene. Radioactivity of ^{125}I was measured on Gamma automat spectrometer Tesla with NaI(Tl) detector (54% efficiency).

Antibody to testosterone was raised in three rabbits immunized by standard procedure [4] with testosterone-carboxymethyloxime-BSA prepared according to Erlanger *et al.* [5]. The steroid–albumin molar ratio assessed from U.V. spectroscopy was 23.

Testosterone was iodinated according to the method of Thakur and Waters [1] modified by one of us (Š.L.). In brief the procedure involved repeated dilution with small amount of methanol of testosterone (100 μg, 0.347 μmol) and Na^{125}I (12.95 MBq (350 μCi), i.e. 0.16 nmol), evaporation of the solution, followed by melting of the mixture at 167 ± 2°C for 2.5 min. To remove excessive testosterone, the product was chromatographed on thin layer silica gel (Merck F-254) in system cyclohexane–ethyl acetate (1:1, v/v) (R_f of testosterone = 0.33). From three products associated with radioactivity and very likely identical with those reported by Thakur and Waters [1] (areas corresponding to R_f 0.21, 0.61 and 0.79, respectively) only the first (R_f = 0.21) appeared to be immunoreactive. It was rechromatographed in another system (benzene–methanol (4:1, v/v) R_f of testosterone = 0.39). The radioactivity peak found in the area of R_f = 0.23 was eluted with methanol and this radioactive material was used for radioimmunoassay. In three experiments the average yield of purified usable labelled ligand related to radioactive iodide was 16% (14.3–19.5%).

Testosterone was assayed in sheep and calf (both male and female) plasma, following extraction with diethyl ether. The plasma of one year old wethers freed of endogenous steroids by treatment with charcoal [6] was taken as a blank. Testosterone labelled with ^{3}H or ^{125}I (167 Bq i.e. 10000 d.p.m. of each

isotope) was incubated with the dry residue of the ether extract (equivalent 50–100 μl of plasma) and appropriately diluted antiserum (100 μl) in the total vol. 0.3 ml at 4°C for 6–16 h. Phosphate buffer, pH 7.0 containing 0.9% NaCl, 0.1% sodium azide and 0.1% gelatine was used as a diluent. Dextran-coated charcoal (0.025% Dextran T-70, 0.25% Norit A, 0.5 ml) was added, the mixture was shaken on Vortex, left standing for 20 min at 0°C and centrifugated. The radioactivity in 0.5 ml aliquot of supernatant was then measured. All output data were processed on minicomputer Compucorp statistician 445.

RESULTS

The reliability criteria of the method using both radioligands were evaluated as follows:

The titre, defined as the highest final dilution at which more than 50% of the radioactivity present in the system remained bound was found to be 1:48000 for ^{3}H and 1:8000 for ^{125}I labelling. In the latter case this value was still dependent on the dose (1:8000 for the total radioactivity equal to 167 Bq and 1:16000 for total radioactivity 85 Bq, respectively), indicating that a certain amount of nonradioactive testosterone still remained in the labelled material.

The apparent association constants at 0°C for testosterone antibody complex were calculated from saturation curves obtained with both radioligands, using Scatchard plot corrected to non-specific binding [7]. The values $3.4 \times 10^{9}\ l \cdot mol^{-1}$ and $1.9 \times 10^{9}\ l \cdot mol^{-1}$ were found for ^{3}H- and ^{125}I labelled testosterone, respectively.

The specificity of the method was determined in terms of cross-reactions of various steroids with antibody (see Table 1), using log-logit transformation for evaluation of the binding data. It is apparent that there are no considerable differences in specificity between both radioligands. It may indicate that iodine labelling does not alter the functional and sterical determinants which are decisive for the specificity of the steroid–antibody interaction.

Precision of the radioimmunoassay using ^{3}H and ^{125}I tracer was determined as average coefficient of variation obtained by analyzing 8 sheep plasma samples (6 males and 2 females), each plasma being analyzed in twelve parallel determinations. The respective values were 4.8% for ^{3}H and 7.7% for ^{125}I labelling.

Sensitivity, expressed as minimal amount of testosterone added to blank plasma, which can be distinguished from zero at 95% probability level was 6 pg for ^{3}H and 12 pg for ^{125}I labelling, respectively.

Accuracy, expressed as percentage recovery of various amounts (12.5–1600 pg) of testosterone added to normal male sheep plasma (16 samples) averaged 97.2 ± 7.9 and $95 \pm 8.1\%$ for ^{3}H and ^{125}I labelling, respectively. The recovery did not depend on the amount of steroid added in this range.

DISCUSSION

Despite the missing identification of the iodinated testosterone derivative, isolation of which is described herein, it may be used as a radioligand in testosterone radioimmunoassay. For concentrations of testosterone higher than 4 ng/ml the extraction step in the method may be avoided without any loss of precision and sensitivity. The reliability criteria obtained by using ^{125}I testosterone are similar to those attained with ^{3}H-labelled steroid. There is, indeed, some reserve about the possibility of increasing the specific radioactivity of ^{125}I testosterone by further purification, which would lead to higher sensitivity of the assay. This iodination technique is easier and cheaper than that utilizing the derivatives of steroids with iodinated aromatic amines. It seems promising especially in cases where tritiated compounds are not available and where there is enough material of authentic compound, as for instance, in synthetic steroids as progestins, anabolics etc.

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Table 1. Specifity of rabbit anti-testosterone-3-carboxymethyloxime-BSA using ^{3}H and ^{125}I labelled testosterone

Steroid	Labelling ^{3}H (% of cross reaction)	Labelling ^{125}I (% of cross reaction)
Testosterone	100	100
17β-Hydroxy-5α-androstan-3-one	42	43
17β-Hydroxy-19-nor-4-androsten-3-one	13.7	13.6
4-Androstene-3,17-dione	0.41	0.75
17α-Methyl-17β-hydroxy-4-androsten-3-one	<0.1	0.37
17α-Hydroxy-4-androsten-3-one	0.1	<0.1
Progesterone	<0.1	<0.1
3β-Hydroxy-5-androsten-17-one	0.1	<0.1
Oestradiol	<0.1	<0.1
Cortisol	<0.1	<0.1

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